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Studies in the Physiology of Parasitism

XVIII. Pectic Enzymes secreted by *Bacterium aroideae*

BY

R. K. S. WOOD

(*Plant Pathology Laboratory, Imperial College, London, S.W. 7*)

With four Figures in the Text

ABSTRACT

Cell-free filtrates from cultures of *Bacterium aroideae* on a simple synthetic medium contained an enzyme, provisionally termed depolymerase, which rapidly reduced the viscosity of pectin solutions, and protopectinase which macerated slices of potato tuber tissue. These filtrates had little pectin-esterase activity.

The activity of depolymerase was directly proportional to enzyme concentration; the activity of protopectinase was approximately proportional to the square root of the enzyme concentration. Crude solutions were partially purified by acetone or ethanol precipitation; ammonium sulphate was less satisfactory as a precipitating agent.

Enzyme preparations which rapidly reduced the viscosity of pectin and pectate solutions were relatively inactive when assayed for polygalacturonase activity by measuring reducing groups liberated. After prolonged incubation some 20 and 40 per cent. hydrolysis of solutions of pectin and pectate respectively was obtained.

The pH optimum for depolymerase activity was near 9·0, the enzyme was activated by Ca^{++} but not by a number of other cations; the loss of activity following dialysis was largely restored by adding Ca^{++}. The enzyme was rapidly inactivated at temperatures above 60° C. and at pH 2·7.

The properties of protopectinase generally resembled those of depolymerase.

Analysis of the breakdown products following enzyme degradation of pectin and pectate solutions by paper chromatography showed that galacturonic acid was not produced but that a number of other products were formed, including one of fairly low molecular weight.

The differences between the pectic enzymes of *B. aroideae* and those from other sources, and the possible identity of depolymerase, polygalacturonase, and protopectinase are discussed.

I. INTRODUCTION

A NUMBER of papers in this series have dealt with the secretion and properties of pectic enzymes produced by certain facultative plant pathogens. Attention was directed to these particular enzymes in view of the strong evidence that they were involved in the mechanism by which these pathogens attack plants and produce a distinct rotting of the host tissue. The amount of enzyme present under specific conditions was estimated quantitatively by a 'maceration test': discs of suitable tissue (potato tuber, turnip root, cucumber fruit, &c.) and of standard thickness were immersed in solutions of the

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enzyme and the time required for loss of coherence (Reaction Time) of the tissue was noted. The inverse of the reaction time was taken as a measure of enzyme activity.

The maceration test has the obvious disadvantage that it can be standardized only to a limited extent. With discs cut from the same sample of tissue, e.g. medulla of a potato tuber or mesocarp of a cucumber fruit, and with a certain experience on the part of the worker, the lowest and highest reaction times for a series of discs is generally within 20 per cent. of the mean value, the error being greater for shorter reaction times. Assessments of activity may therefore be readily and fairly accurately obtained in any one experiment. Distinct differences in reaction times may, however, be shown between discs taken from different potato tubers, tubers of different varieties, or tubers of the same variety at different times of the year. No absolute comparison is therefore possible between the results of different experiments if natural substrates are used. A further drawback of the method is that with such a complex substrate any attempt to study the reaction in biochemical terms is excluded. The need for a more defined substrate is obvious and was in fact pointed out at an early stage in these investigations (Brown, 1915). The need became greater still when it was shown that the macerating enzymes of different plant pathogens were not identical, differing, for example, in their pH optima and resistance to heating (Chona, 1932; Menon, 1934; Talboys, 1950).

For analysis of these and other differences it became necessary to employ some of the techniques which have been introduced in recent years for the investigation of pectic substances and the enzymes acting upon them. Several workers in this laboratory have made studies along these lines with a variety of plant pathogens. As the background of this work, both as regard substances and terminology, may not be familiar to some mycologists and plant pathologists, it will be fitting to give as a preface to this, the first paper of a group, a summary of the modern views on the chemistry of pectic substances and on the different kinds of pectic enzymes. For fuller accounts the reader is referred to the comprehensive monograph of Kertesz (1951) and to recent reviews (Hinton, 1951; Kertesz and McColloch, 1950; Lineweaver and Jansen, 1951).

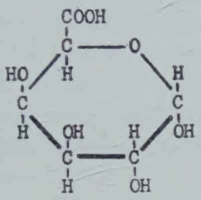
II. PECTIC SUBSTANCES AND ENZYMES

Higher plant tissue may contain a relatively large proportion of pectic substances most of which is found in the cell-wall. The limits of the term *pectic substances* are still not clearly defined. It is now established, however, that they are for the most part composed of residues of galacturonic acid (Fig. 1*a*) linked through positions 1 and 4 of the pyranose ring to give a polygalacturonic acid (Fig. 1*b*). These residues are thought to be combined mainly in linear chains; more complex structures, including ring formations, have also been suggested. The minimum molecular weight at which a number of combined residues become a pectic substance is largely a matter of definition. A commonly accepted limit is that at which colloidal solutions are produced.

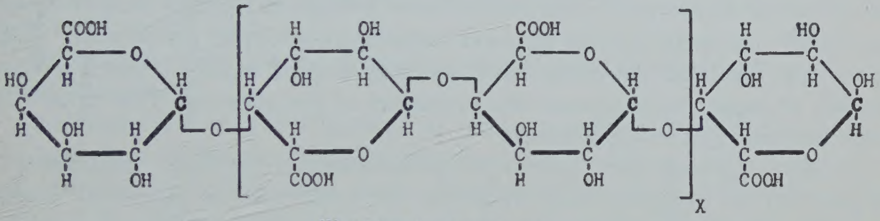
A further characteristic of some pectic substances is that the carboxyl groups of the galacturonic acid residues may be esterified with methyl alcohol (Fig. 1*c*), or may form salts with various cations.

The nomenclature of the pectic substances is still not settled; a summary of the terminology generally adopted at present is as follows:

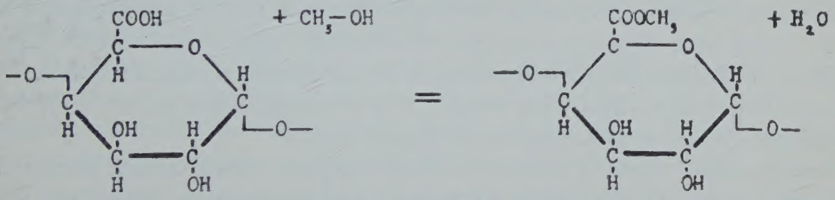
Pectic acid is the term applied to a pectic substance mostly composed of colloidal polygalacturonic acid and essentially free from methyl ester groups. The salts of pectic acids are either normal or acid pectates.



(*a*) α-galacturonic acid (pyranose form).



(*b*) Polygalacturonic acid.



(*c*) Esterification of unit of polygalacturonic acid chain with methanol.

FIG. 1. Structure of certain pectic substances and derivatives.

Pectinic acid is the term used for a colloidal polygalacturonic acid containing more than a negligible proportion of methyl ester groups. The salts of pectinic acids are either normal or acid pectinates.

Pectin is a general term applied to a pectinic acid with a relatively high methyl ester content and which is capable of forming a gel with acid and sugar under suitable conditions.

Protopectin is the term applied to the water-insoluble parent substance or substances which occur in plants and which upon restricted hydrolysis yield pectinic acids.

The early stages of parasitic attack probably involve degradation of protopectin if this term be used to include the insoluble pectic substances of the middle lamella. The ill-defined nature of this substrate makes detailed investigation of its breakdown by pectic enzymes very difficult. Its composition is still largely a matter of conjecture; evidence for the existence of linkages other than the 1,4-glycosidic bonds between individual galacturonic acid residues is, however, accumulating. These may take the form of bridging by multivalent metallic ions, particularly calcium, between carboxyl groups of adjacent chains, association through esterfied phosphoryl groups or with cellulose or proteins. Each of these would be expected to decrease the solubility of the parent pectic substance. There remains the possibility, however, that the insolubility of the pectic components of protopectin is primarily a function of their very high molecular weight as simple linear polymers.

While some stability has been reached in the terminology of pectic substances, the nomenclature of the pectic enzymes is still in a state of flux. The following two are well defined although there is still some doubt as to their precise mode of action.

Pectinesterase (syn. pectase, pectin-methylesterase) is the enzyme which catalyses the hydrolysis of the methyl ester groups in pectinic acids to yield methanol and pectinic acids of lower methoxyl content or pectic acid. This enzyme is produced by many fungi and may also be readily isolated from a variety of higher plant tissues, the properties of the enzymes from these two sources being somewhat different.

Polygalacturonase (syn. pectin-polygalacturonase, pectinase, pectolase) is applied to the enzyme which catalyses the hydrolysis of glycosidic bonds between galacturonic residues in pectic substances and thereby gives polygalacturonic acids of decreasing molecular size and, finally, monogalacturonic acid. It has been shown (Jansen and McDonnell, 1945) that the rate of breakdown of the molecular chain increases as the methoxyl content decreases. The enzyme is produced by a wide variety of micro-organisms; as yet there is no conclusive evidence that it occurs in higher plants.

Depolymerase. Less is known of other enzymes which attack pectic substances. In recent years some have been described which differ from polygalacturonase in not producing so complete an hydrolysis of various pectic substrates (McColloch and Kertesz, 1948; Luh and Phaff, 1951; Roboz et al., 1952; Seegmiller and Jansen, 1952). These enzymes have been given a variety of names; in this and subsequent papers they will be referred to as depolymerases.

Protopectinase. The independent existence of this enzyme is still conjectural. It is the term applied to the enzyme or group of enzymes which by their action bring into solution the protopectin of the cell-wall. When the substrate is plant tissue in the natural state the effect is to produce *maceration* or the separation of cells from each other, together with greater or less destruction of the cell-wall proper. So far the enzyme has been demonstrated only by its action on plant tissues and the types of chemical reactions involved must remain hypothetical until more is known of the nature of protopectin itself. Protopectinase has been regarded by some workers as identical with polygalacturonase or depolymerase; there is no *a priori* reason why this should not be so if in fact protopectin consists essentially of polygalacturonic acid chains of variable methoxyl content. It is possible, however, that there are enzymes which have pectic materials of high molecular weight as their specific substrates or which act upon linkages between such substances and other cell wall components. Experimental evidence for this is already available (Davison and Willaman, 1927; Beavan and Brown, 1949).

III. MEASUREMENT OF ENZYME ACTIVITY

Methods for estimating the activity of certain enzymes described above are now reasonably standardized; those used for others are still largely empirical. Methods in general use are summarized below.

Pectinesterase activity is now generally estimated by measuring the methanol liberated or the increase in free carboxyl groups during de-esterification of pectin or pectinic acids at constant pH. The estimation of free carboxyl groups is the simpler method, is reliable under standard conditions, and is the one now most widely used.

Polygalacturonase. Of the methods available, the following have been used most extensively.

(*a*) Measurement of the increase in reducing power (as aldehyde groups) after rupture of the glycosidic linkages between adjoining units of the polygalacturonic acid chain. This method has the advantage that it measures the product of a chemical reaction: it is more accurate in the later stages of hydrolysis and is less useful for the earlier stages which are probably of greater significance in plant pathological studies.

(*b*) Determination of viscosity changes which are readily and quickly determined in the early stages of breakdown of pectic substances in solution. The main difficulty here lies in the interpretation of the data as there is no simple relation between loss of viscosity and decrease in molecular size.

Protopectinase. The activity of this enzyme is most conveniently measured with slices of plant tissue. The simple methods introduced by Brown (1915) are still those generally used. Slices of standard size and thickness are immersed in enzyme solutions; the end-point of the reaction is taken when the tissue shows no resistance to pulls from opposite sides.

Some of the methods outlined above were used in investigations of properties of solutions of pectic enzymes prepared from the soft-rot organism, *Bacterium aroideae.* A summary of part of this work has already appeared (Wood, 1951). A fuller account now follows.

IV. MATERIALS

The isolate of *Bacterium aroideae* (Townsend) Stapp., [*Erwinia aroideae* (Townsend) Holland] used was obtained from the Stock Culture Collection, Bacteriology Department, Imperial College. It was originally isolated from tobacco. The organism was first described as causing a soft rot of the corm, petiole, and flower-stalk of the calla lily (Townsend, 1904). It has now been recorded on a wide range of angiosperm species (Elliott, 1951) and is generally regarded as a strain of *Bacterium carotovorum* (Jones) Lehmann, differing from the latter in not forming gas from certain carbohydrates (Dowson, 1949). The isolate was very pathogenic to potato tubers, producing a rapidly spreading soft-rot a few days after inoculation and incubation at 25° C. Cultures were maintained on a potato glucose agar.

Samples of pectic materials were kindly supplied by the California Fruit Growers Exchange, Ontario, California. These were further purified by extraction with 60 per cent. ethanol acidified with hydrochloric acid at a final concentration of 5 per cent. washing with 60 per cent. ethanol until free of chloride, rinsing with 95 per cent. ethanol and quickly drying at 60° C. After purification in this way the sample of pectin used had a methoxyl content of 9·2 per cent. The Zeisel method was used for this determination after taking precautions to remove adsorbed ethanol.

Pectin solutions were prepared by dissolving the dry powder in cold water with constant stirring. They were stored under toluene at 3–4° C. with little change in viscosity over a period of 1–2 months.

Solutions of sodium pectate were prepared either by neutralizing pectic acid or by de-esterifying pectin solutions with 0·1 N NaOH. In both cases solutions were kept at 18–20° C. during preparation and the pH was not allowed to rise above 10·0. Even so, some degradation occurred, 0·5 per cent. solutions of sodium pectate having viscosities some 50 per cent. lower than those of a 0·5 per cent. solution of pectin.

For maceration tests, strips of potato tuber tissue, 0·5 mm. thick, were used. These were cut on a sliding microtome from blocks of medullary tissue made fully turgid by injecting with distilled water under the air pump. The strips were washed with distilled water, and the surface dried between layers of filter paper before immersing in test solutions.

V. PREPARATION OF ENZYME

The following medium was found particularly suitable for growth and secretion of macerating and other pectic enzymes by the organism:

Glucose	1·0%
Asparagine	0·25%
Potassium dihydrogen phosphate	0·1%
Magnesium sulphate ($7H_2O$)	0·05%
Calcium carbonate	1·0%

The calcium carbonate prevented the acid drift which would otherwise have occurred in this medium and which curtails the growth of the organism. Forty ml. of the medium were placed in 10-oz. medicine bottles and after sterilization inoculated with 1 ml. of a 24-hour culture of the bacterium on the same medium. The cultures were incubated at 25° C. for 4 days. They were then shaken with a few drops of toluene, stored overnight, and the bacteria removed the following day by high-speed centrifuging for 5 minutes. Solutions were kept under toluene at 3–4° C. for short periods and at −20° C. for longer periods. Activity slowly decreased at 3–4° C., but at 20° C. there was little if any loss over periods of months. The pH of these solutions was in the range of 7·6–8·0.

VI. Determination of Enzyme Activity

Pectinesterase. Activity was estimated in the first place by determining the methanol liberated from solutions of pectin by crude preparations. The substrate was as follows: 1 per cent. pectin solution, 5 vol.; veronal buffer (Michaelis, 1930) at pH 8·0, 2 vol.; 0·01 M $CaCl_2$, 2 vol; crude filtrate, 1 vol. The mixture was incubated at 25° C. Samples were removed at intervals over a period of 3–4 hours and their methanol content determined by the colorimetric method of Bertrand and Silberstein (1948) which was sufficiently sensitive under the conditions of the experiments to detect a 5 per cent. conversion of the total methoxyl content of the sample. Determinations of known quantities of methanol added to certain samples gave figures corresponding to 90 per cent. of the theoretical value. Methanol was also readily detected in solutions of sodium pectate derived from the same sample of pectin by alkaline de-esterification.

No methanol was, however, detected in any samples taken from the pectin solution to which had been added the crude enzyme preparation. Since loss of viscosity of a sample of the same mixture was almost complete 20 minutes after adding the enzyme, it was clear that this had occurred in the absence of or with very low pectinesterase activity.

This was confirmed by the following experiment similar to many others carried out. To 5 vol. of an unbuffered 1 per cent. solution of pectin at pH 8·0 was added 5 vol. of a crude enzyme solution before and after autoclaving, and 0·1 vol. of a phenol red solution. Both were kept at 25° C. for 2 hours. There was little change in the colour of the mixture containing the autoclaved preparation; the change of colour of the mixture containing the untreated enzyme corresponded approximately to the conversion of 2 per cent. of the total methoxyl groups and again indicated a very low level of pectinesterase activity.

From these results it was concluded that crude solutions, i.e. crude filtrates from cultures of *B. aroideae*, are practically free of pectinesterase.

Polygalacturonase and depolymerase. Early in this work it was found that solutions which rapidly macerated plant tissues gave, when tested on solutions of pectin or sodium pectate, relatively little increase in reducing power, even after prolonged incubation. For viscometric study, pectin solutions were chiefly used as substrates because the enzymes secreted by *B. aroideae* were fully active only in the presence of calcium and this introduced complications with solutions of soluble pectates. Moreover, some degradation occurred during the preparation of sodium pectate from pectin and since, from a plant pathological point of view, it is the attack of the higher molecular weight compounds which is probably of greatest significance, it seemed desirable to use substances of as high a degree of polymerization as possible.

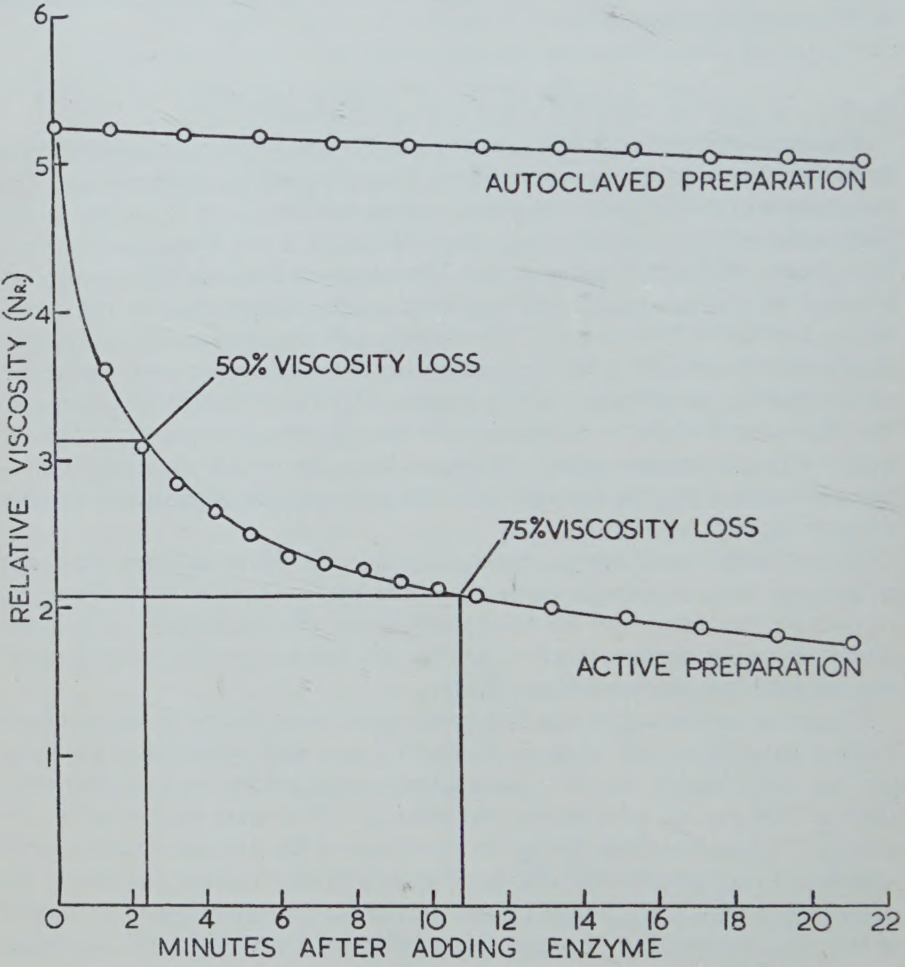


FIG. 2. Effect of *B. aroidaea* preparations on viscosity of pectin solutions.

Reproducible results with viscometric methods are obtained only if the experimental procedure is rigidly standardized. The technique adopted was as follows. To 5 ml. of a 1 per cent. solution of pectin (or, less frequently, sodium pectate) was added sufficient 0·1 N NaOH to bring the pH to a required value and 2 ml. of a suitable buffer solution at the same pH. A Michaelis veronal buffer was used, primarily because of the solubility of its calcium salts. The mixture was made up to 9 ml. and placed in a water-bath at 25° C. After a few minutes 1 ml. of an enzyme solution at 25° C. was transferred to the pectin solution and 5 ml. of the final mixture rapidly transferred to an Ostwald viscometer already at 25° C. The first viscosity determination was made 1 minute after adding the enzyme solution. Similar readings were taken at suitable intervals, generally over a 20-minute period. Relative viscosity (water = 1·0) was plotted against time (t) at which viscosity was determined. These values are difficult to obtain accurately because the measurement of the viscosity may take an appreciable time during which the viscosity is changing, sometimes quite rapidly, e.g. immediately after adding an active enzyme preparation. The following approximations were therefore made. If x be the time between adding the enzyme to the mixture and the start of any viscosity measurement and y the time of flow through the viscometer and z the time of flow of water through the viscometer, then $nr = y/z$ and $t = x + y/2$.

From curves of nr and t may be obtained the time taken for a preparation of unknown activity to produce a given viscosity change in a standard solution. The end-points generally chosen were those corresponding to 50 or 75 per cent. of the total possible loss of viscosity, the lower limit being the viscosity of water and the upper limit that of the substrate in the absence of enzyme. Fig. 2 shows a typical curve obtained with active and inactivated preparations. With the active preparation, a 50 per cent. loss of viscosity occurred after 2·4 minutes and a 75 per cent. loss after 10·8 minutes. The curve for the same preparation after autoclaving shows that there was only a slight loss in viscosity after 21 minutes' incubation. The relationship between enzyme concentration and activity as measured by the ability to reduce the viscosity of standard solutions was studied by this method. Aliquots containing different proportions of untreated and autoclaved enzyme preparations were added to standard volumes of the substrate described above at pH 8·0. The concentration of thermostable substances was thus the same for each determination; this is important in view of the effect of certain salts on the viscosity of pectin solutions. Curves of nr and t were obtained for each concentration of active enzyme and from them, activities, expressed as the reciprocal of the times required for the solutions to show a 50 or 75 per cent. loss of viscosity, were determined. Graphs of activity against enzyme concentration are shown in Fig. 3 and show clearly that for these end-points and range of enzyme concentrations activity was directly proportional to concentration.

The relation between viscosity change and increase in reducing power of solutions is brought out by the following experiment, the substrates being 0·5 per cent. solutions of pectin or of sodium pectate derived from the former. Both were kept at pH 8·0 throughout the reaction with a veronal buffer. The enzyme used was a partially purified dialysed preparation concentrated twentyfold by acetone precipitation from a crude solution. It was used at a concentration of 5 per cent. of the final volume, i.e. at approximately ten times the concentration used in the viscosity tests described in connexion with Fig. 2. The mixtures were incubated at 25° C. under toluene. Viscosity determinations were made at frequent intervals during the early stages of the reaction

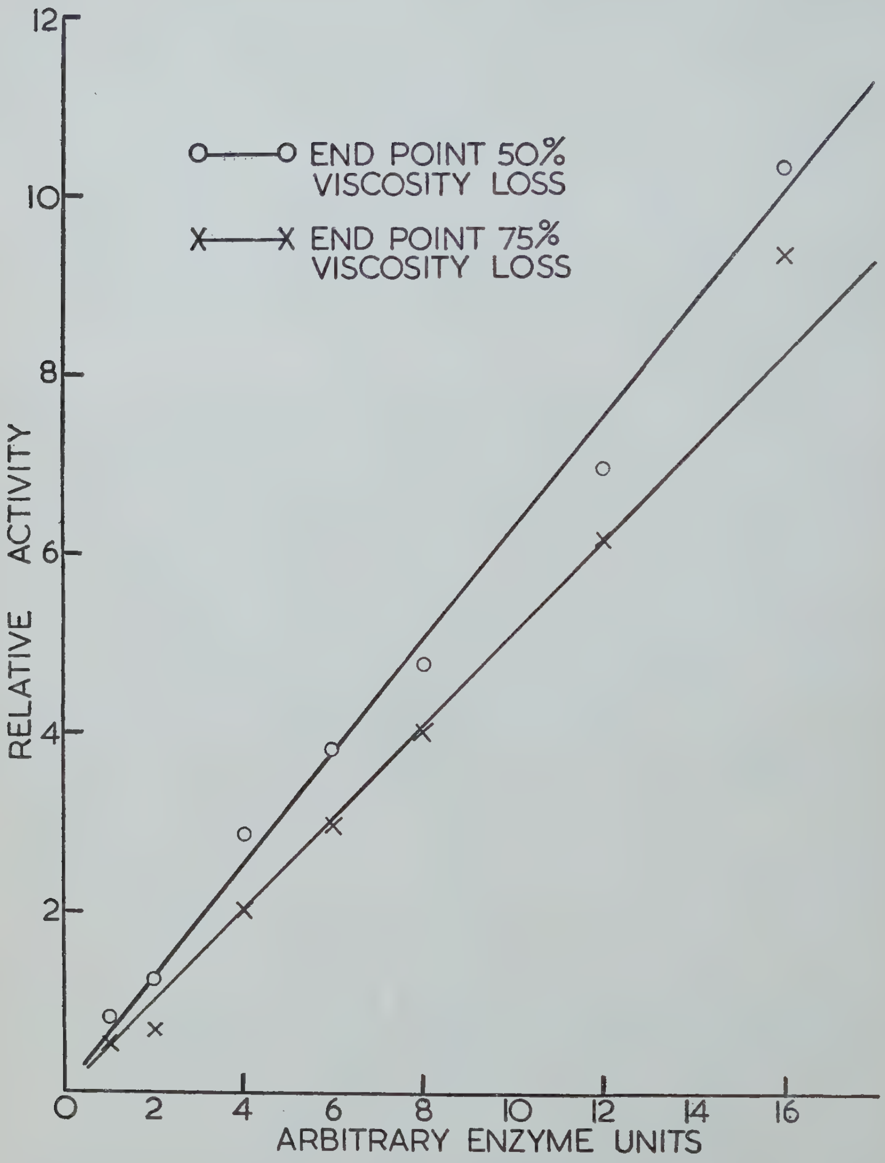


Fig. 3. Relation between activity and concentration of enzyme in *B. aroideae* preparations.

and the reducing power of samples of the solutions measured by the Jansen and McDonnell (1945) modification of the Willstatter–Schudel hypoidite method. The reducing power was calculated in terms of galacturonic acid (1 ml. of 0·1 N iodine reduced is equivalent to 9·70 mg. anhydrous galacturonic acid). Known quantities of galacturonic acid added to samples of the mixtures gave increases in reducing power equivalent to 95 per cent. of the theoretical value.

The results are set out in Table I. Increase in reducing power has been expressed as a percentage of the theoretical value for complete hydrolysis of the materials; viscosity loss as a percentage of the total possible loss.

The outstanding result, as seen for example with pectin, is the rapid loss of viscosity accompanied by a very slow rate of hydrolysis as measured by increase in reducing power. Thus in 10 minutes, with the concentrated enzyme solution used, almost all the viscosity ascribable to pectin had disappeared whereas only about 5 per cent. hydrolysis had taken place. Even after 24 hours the percentage hydrolysis was only about 20, and there was hardly any increase beyond this figure after a further 6 days even when more enzyme was added. The preparation of sodium pectate behaved similarly, although here the discrepancy between hydrolysis and viscosity change was not as striking.

TABLE I

Increase in Reducing Power in Relation to Viscosity Loss

	% hydrolysis.			% viscosity loss.	
Time (min.).	Pectin.	Sodium pectate.	Time (min.).	Pectin.	Sodium pectate.
5	1·7	10·0	1	81	57
15	6·7	11·7	2	87	65
30	8·3	14·1	4	92	82
60	11·7	16·6	6	95	88
120	12·4	20·4	10	97	92
480	15·0	26·6	14	97	93
1,440	19·1	37·4	20	98	96

The reducing powers of the final solutions, corresponding to 20 per cent. (for pectin) and 40 per cent. (for sodium pectate) of the theoretical figure for complete hydrolysis, are much lower than those quoted for the polygalacturonases of certain fungi. For these, values of 70–90 per cent. have been obtained. The behaviour of enzyme preparations from *Bacterium aroideae* is similar to that described by Beavan and Brown (1949) for *Byssochlamys fulva*, by Roboz et al. (1952) for *Neurospora crassa*, by Luh and Phaff (1951) for various yeasts, and by McColloch and Kertesz (1948) for tomato fruit.

In view of these results, it is clear that the viscosity-reducing enzyme of *B. aroideae* cannot be called polygalacturonase according to the definition at present accepted. It does not break down pectin to galacturonic acid to any extent if at all (see later). Furthermore, the fact that the viscosity of pectin is rapidly destroyed in the nearly complete absence of pectinesterase is an additional argument against equating it with polygalacturonase since the substrate of the latter is held to be pectate and not pectin. The viscosity-reducing enzyme of *B. aroideae* has properties resembling more those of a *depolymerase* and will be so named throughout this paper.

Protopectinase. Activity of this enzyme was measured by a modification of the disc method mentioned earlier. Strips of tissue measuring 45 × 7 × 0·5 mm. were cut from sheets obtained from a block of the medulla of a potato tuber which had been soaked in water until fully turgid. The strips were well washed and injected with water before use. The ends were lightly clamped together and the loops so formed suspended in a test solution. A glass hook to which was attached a weight (total weight 10 g.) was slipped through the loop from time to time and the end-point (reaction time) taken as the time at which the hook broke through the tissue. The hook had a straight length in contact with the tissue; this was held horizontally when the tests were made. An essentially similar method was used by Davison and Willaman (1927).

TABLE II

Effect of Enzyme Concentration on Protopectinase Activity

Enzyme concentration (arbitrary units).	Activity.		
	Expt. 1.	Expt. 2.	Mean.
100·0	55	54	55
50·0	43	38	40
25·0	33	28	30
12·5	26	21	24
6·3	16	13	15
3·2	13	10	12
1·6	9	8	9
0·8	5	7	6

The effect of concentration on activity was determined in the following experiments. Different volumes of untreated and autoclaved enzyme solutions were mixed to give appropriate concentrations of active enzyme. Twelve per cent. by volume of a veronal buffer at pH 8·4 was added to each and the reaction time estimated as described above. Table II shows the results obtained with two different preparations; six strips were used in each test and the mean reaction time calculated. The undiluted preparations were assumed to contain 100 arbitrary enzyme units per unit volume. Activity is expressed as 1000/reaction time in minutes.

Fig. 4 shows graphs of the means of the activities obtained in the two experiments plotted against (*a*) the enzyme concentration and (*b*) the square root of the enzyme concentration. It is seen that activity is approximately proportional to the square root of the enzyme concentration. This relationship must be borne in mind in interpreting data to be presented later in which activity is expressed as the reciprocal of the reaction time. A similar relationship has been noted for the enzymes secreted by *Botrytis cinerea* (Brown, 1915).

VI. PARTIAL PURIFICATION OF ENZYME SOLUTIONS

Undiluted crude cell-free preparations from 4-day cultures macerate slices of potato tuber tissue 0·5 mm. thick in 12–15 minutes and, in ten-fold dilution, bring about almost complete loss of viscosity of 0·5 per cent. solutions of pectin in 20 minutes. Partially purified preparations may readily be obtained from these crude solutions by fractional precipitation with acetone. The optimum pH for precipitation was determined by adding equal volumes of acetone at −18° C. to volumes of a crude preparation at 11° C. brought to and maintained at various pH values with 0·1 N NaOH or HCl. At pH 7·2 and above the precipitate flocculated readily and was easily collected by low-speed centrifugation. Below pH 6·2, much lighter precipitates were formed and higher speeds were required. After removal of excess acetone the precipitates were taken up in water and their activity determined by viscosity and maceration methods. In each case calcium chloride (final concentration 0·002 M) was added and activity measured at pH 8·0–8·2. With both methods

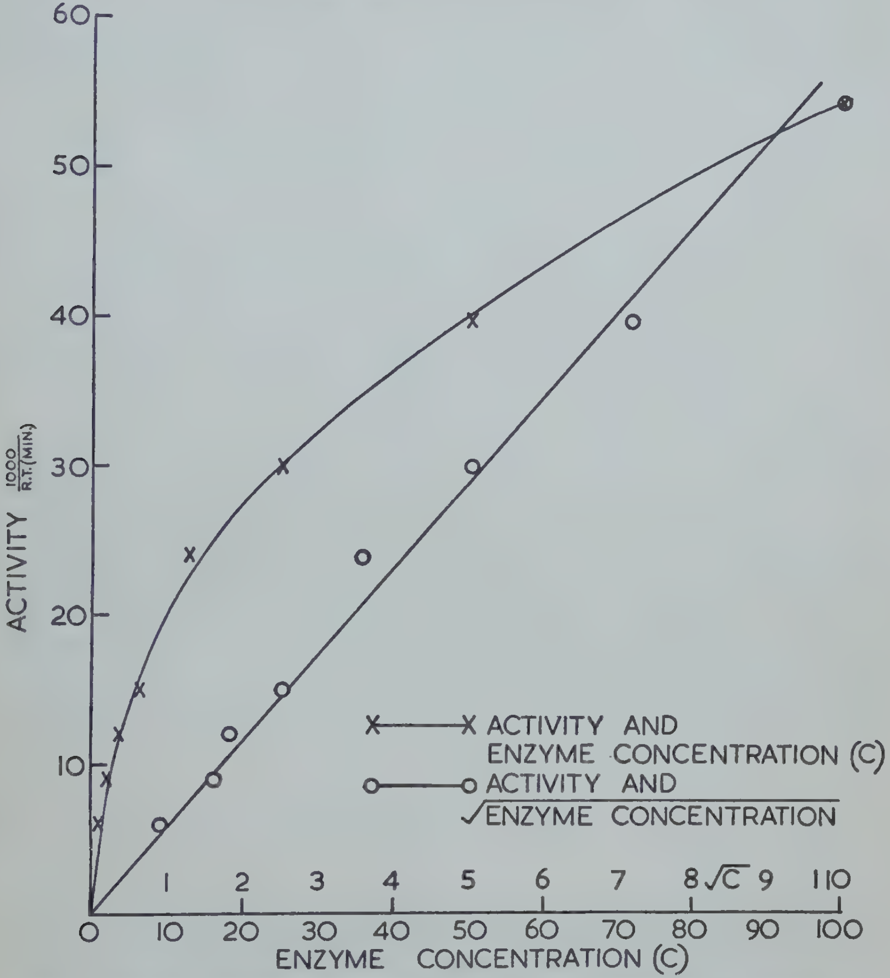


FIG. 4. Relation between macerating activity and protopectinase concentration in *B. aroideae* preparations.

precipitates produced at or below pH 5·0 had low activity. This increased rapidly above pH 7·2 and reached a maximum in the region of pH 9·0, thereafter decreasing. At the optimum pH some 60–70 per cent. of the original activity was recovered.

At pH 9·0 both depolymerase and protopectinase activity of precipitates increased as the acetone concentration was increased, reaching a maximum at 50 per cent. Above this concentration there was no increase but precipitates collected from 90 per cent. acetone solutions were much less active.

Ethanol was not found as suitable as a precipitating agent. Again, maximum recovery was obtained at high pH values (*c.* 9·0) and at final concentrations of 50 per cent. Recovery was, however, only of the order of 30–40 per cent.

Ammonium sulphate was also found to be not as suitable as acetone. Concentrations of 70–80 per cent. saturation were required before precipitation occurred even at low temperatures and considerable difficulty was experienced in collecting the precipitates by centrifuging. It could, however, be collected by filtration through Hyflo Super-Cel and then brought into solution by elution with 0·5 M NaCl. The salt was not as convenient a precipitating agent as acetone which was used in all subsequent work.

Solutions obtained after precipitation with acetone were generally dialysed before use. After dialysis the dry weight content of solutions was quite low. Thus, in one preparation, the precipitate from a litre of crude solution was taken up in 25 ml. of water, the insoluble part of the precipitate discarded, and the supernatant solution dialysed against 0·002 M calcium chloride (see later). After allowing for the latter the dry weight of the final solution was approximately 0·1 per cent. This solution could be diluted forty times and still macerate strips of potato tuber tissue 0·5 mm. thick in 15–20 minutes; diluted 400 times, almost complete loss of viscosity of 0·5 per cent. pectin solutions was obtained in 20 minutes.

VII. PROPERTIES OF ENZYME SOLUTIONS

(*a*) *Depolymerase*

1. *Effect of pH.* This was first determined with a 0·5 per cent. pectin solution as substrate. One ml. of a partially purified preparation was added to 9 ml. of a standard pectin solution after this had been adjusted to various pH values with NaOH. The viscosity of the solutions was measured at intervals over a 20-minute period during which there were changes of pH in all mixtures. These were slight below pH 8·0 but above this value were substantial, amounting to as much as 0·6 of a unit at the highest values used. Activities were estimated as the inverse of the time taken for the solutions to reach certain end-points since it has already been shown that activity measured in this way is proportional to enzyme concentration. End-points representing 25 or 50 per cent. viscosity loss were chosen in order to obtain estimates of activity at unfavourable pH values where the reduction of viscosity was slow.

Activities at different pH values are shown in Table III.

There is general agreement between the estimates of activity at the two end-points and it would seem that the optimum pH is in the neighbourhood of 9·0. However, the use of pectin at high pH values raises the following difficulties. Above pH 7·0, de-esterification occurs in the absence of enzyme, the rate rapidly increasing at higher values. Since *fungal* polygalacturonase activity increases as the methoxyl content of the pectic substrate decreases (Jansen and McDonnell, 1945), it was possible that the high activity at pH values above 8·0 was in part due to this factor, particularly as crude solutions contained little or no pectinesterase. In addition, above pH 8·4 some reduction in viscosity occurred in the absence of enzyme. Again, this was greatest at high pH values. Thus, at pH 10·0, the relative viscosity of a 0·5 per cent. solution fell from an initial value of 5·3 to 4·7 after 20 minutes at 25° C.

TABLE III

Effect of pH on Depolymerase Activity with Pectin as Substrate

	Relative activity	
pH.	25% viscosity loss.	50% viscosity loss.
6·0	<3	<3
7·0	<3	<3
7·7	14	11
8·6	73	60
9·0	100	100
9·5	47	60
10·0	37	54

TABLE IV

Effect of pH on Depolymerase Activity with Sodium Pectate as Substrate

	Relative activity	
pH.	25% viscosity loss.	50% viscosity loss.
6·1	<6	<11
7·0	<6	<11
7·6	<6	<11
8·3	33	11
8·7	39	32
9·3	100	100
10·2	26	11

To overcome these difficulties the above experiments were repeated with solutions of sodium pectate derived from the same sample of pectin by alkaline de-esterification. With this substrate there was little change of viscosity in the absence of active enzyme even at the highest pH values used. However, the low initial viscosities of 0·5 per cent. solutions was a disadvantage. The data given in Table IV were obtained in the same way as those of Table III.

The results are in general agreement with those obtained with pectin and show that the pH optimum is in the region of 9·0. Below pH 7·0, activity was less than a twentieth of that at the optimum. While a definite decrease in activity was obtained above pH 9·0 with pectin as a substrate, its magnitude was difficult to assess in view of the degradation which occurred in the absence of enzyme. With sodium pectate, this difficulty did not arise and a pronounced reduction of activity took place above pH 9·3.

In view of the possible direct effect of high pH values in de-esterifying pectin, estimates of activity with this material were made not at the optimum pH but at pH 8·0–8·3; activity was still high at these values, but de-esterification was relatively slow.

2. *Effect of dialysis and cations.* Solutions obtained from crude cell-free preparations were dialysed in cellophane tubes slowly rotated in a large volume of water at 1° C. This treatment if sufficiently prolonged always resulted in a marked loss of activity. This could, however, be largely restored by adding to the enzyme and substrate mixture a volume of an autoclaved undialysed enzyme solution. Analysis of this effect showed that the active component was the calcium ion derived from the calcium carbonate added to stabilize the pH of growing cultures. This has been confirmed many times in experiments similar to the following. Two ml. of mixtures containing equal volumes of a dialysed enzyme solution and solutions of calcium chloride at various concentrations were added to a substrate of pectin and veronal buffer at pH 8·3. The final concentration of pectin was 0·5 per cent. It was necessary to add the enzyme and calcium chloride simultaneously to offset the increase of viscosity which occurred when this salt was added to the solution of pectin in the absence of the enzyme. Even when this was done there was a temporary increase in viscosity at the highest concentrations of calcium chloride which were used. The relative viscosities of the different mixtures determined at intervals over a 22-minute period are given in Table V.

TABLE V

Effect of Calcium Chloride on Depolymerase Activity with Pectin as Substrate

Enzyme solution	Relative viscosity Minutes after adding enzyme.								
	1	3	5	7	9	14	16	20	22
Original	4·5	2·9	2·3	2·0	1·8	1·7	1·6	—	1·5
Dialysed	4·3	4·2	—	—	—	4·1	—	—	3·9
,, +0·004 M $CaCl_2$*	(9·8)	(6·5)	3·7	2·8	2·3	1·9	1·8	1·7	1·6
,, +0·002 M $CaCl_2$	(5·5)	4·0	3·1	2·7	2·3	2·0	1·9	1·8	1·7
,, +0·001 M $CaCl_2$	4·1	3·4	3·0	2·7	2·5	2·2	2·1	1·9	1·8
,, +0·0002 M $CaCl_2$	3·9	3·6	3·4	3·2	3·0	2·9	2·8	2·6	2·5

* Represents final concentration of $CaCl_2$ in terms of $CaCl_2$ *added* and does not include that already present.

The effect of calcium chloride in restoring the activity of dialysed preparations is clear. With the highest concentrations used, 0·004 M, although interpretation of the data for the early stages of the reaction is complicated by the direct effect of calcium in increasing viscosity of the solutions, later readings were little below those obtained with the original solution. A pronounced effect was obtained even with concentrations of calcium chloride as low as 0·0002 M.

In a series of similar experiments the effects of the chlorides of sodium, magnesium, strontium, barium, cobalt, manganese, and ferrous iron were tested over a range of concentrations. In no case were results obtained comparable to those with calcium. The chloride ion may therefore be eliminated as an activating agent and the whole of the effect attributed to the calcium ion. The ineffectiveness of barium and strontium is rather surprising in view of their chemical similarity to calcium and their similar behaviour with pectic substances.

As a corollary to these findings, there was little loss of activity when enzyme preparations are dialysed against 0·002 M solutions of calcium chloride.

TABLE VI

Effect of Calcium Chloride on Depolymerase Activity at Different pH Values with Pectin as Substrate

pH.	Dialysed enzyme. Relative viscosity after 20 min.	Dialysed enzyme $+0·002$ M $CaCl_2$. Time taken to reach viscosity in column 2.	Ratio of activity: $\frac{\text{Enzyme}+CaCl_2}{\text{Enzyme}}$.
7·0	3·8	2·2	9·1
7·5	3·5	2·1	9·5
8·0	3·2	1·3	15·4
8·5	2·8	1·3	15·4
9·0	2·5	1·9	10·5

Another aspect of this effect of calcium was observed during purification by precipitation with acetone from crude preparations. These precipitates often showed a marked loss of activity when taken up in water and used as such. By adding calcium, however, most of the original activity was recovered. As a general practice, therefore, partially purified preparations were used only in the presence of added calcium.

The effect of calcium was also studied at different pH values. In one series a dialysed enzyme solution was used and, at different pH values, the relative viscosity of the mixtures determined 20 minutes after adding the enzyme. In another series and at similar pH values but in the presence of 0·002 M calcium chloride the times taken for corresponding mixtures to reach these viscosities was estimated. These data are given in Table VI.

A 9–15-fold increase was obtained at the different pH values.

The effects of dialysis were simulated by treating a crude enzyme solution with a cation absorbing ion-exchange resin. The resin was brought to the sodium form with 0·1 N NaOH, well washed with ion-free water and adjusted to pH 8·4 with phosphate buffer. The resin was then added to a volume of a crude preparation which was gently agitated for 30 minutes. The supernatant was decanted and tested as such, and also in the presence of calcium chloride. A 0·5 per cent. of pectin solution at pH 8·0 was used as substrate.

The data shown in Table VII are based on a 50 per cent. viscosity loss as end-point.

The loss of activity following removal of cations from the crude enzyme solution was, in large measure, restored by the addition of calcium chloride.

Further experiments showed that activation by calcium did not depend on the presence of the ion in the culture fluid during growth of the bacterium and secretion of the enzyme. The bacterium was cultured in a medium containing glucose 1 per cent., asparagine 0·25 per cent., magnesium sulphate ($7H_2O$) 0·03 per cent., to which was added phosphate buffer at pH 7·0 in different concentrations. These cultures were compared with those on a normal medium containing 0·0073 M phosphate and excess calcium carbonate. The buffers were used at final concentrations of 0·067, 0·048, 0·029, and 0·014 M; they did not prevent a drift in the pH of the culture fluids to lower values during growth of the bacteria, the final values being pH 6·5, 6·3, 5·9, and 5·6. Cell-free solutions from the cultures were tested by adding 1 ml. to a standard pectin substrate with and without calcium chloride at a final concentration of 0·002 M. In each case the addition of calcium caused an approximate threefold increase in relative activity. Comparable figures were obtained with these solutions after partial purification by acetone precipitation.

TABLE VII

Effect of Treatment with an Ion-exchange Resin on Depolymerase Activity with Pectin as Substrate

Treatment.	Relative actity.
Original solution	100
After resin treatment	9
,, ,, ,, $+0{\cdot}002$ M $CaCl_2$	73

It is not easy to conduct experiments similar to the above with sodium pectate solutions owing to the rapid formation of a precipitate of calcium pectate when solutions containing calcium are added. However, some interesting results were obtained when solutions of sodium pectate, calcium chloride, and dialysed enzyme were mixed in various combinations. These are summarized below.

(*a*) (Sodium pectate+calcium chloride)+(dialysed enzyme). The first reaction led to the formation of a gel. It was then difficult to mix the enzyme solution with this gel which was broken down only very slowly.

(*b*) (Sodium pectate+dialysed enzyme)+(calcium chloride). In the absence of calcium, breakdown of the sodium pectate was slow and the addition of calcium shortly afterwards gave a soft gel which again disintegrated only slowly.

(*c*) (Sodium pectate)+(dialysed enzyme+calcium chloride). There was slight gel formation when these were mixed. The gel rapidly disappeared and the viscosity decreased rapidly.

The results suggest that calcium is necessary for full activity and that in its presence molecular chains of sodium pectate are rapidly broken down to units not large enough to form a precipitate on the addition of calcium. Alternatively, one might suppose that the calcium required for full enzyme activity was made unavailable in some way for the formation of a calcium pectate precipitate.

TABLE VIII

Effect of Depolymerase on Viscosity of Pectin Acetate Solutions

Enzyme solution	Relative viscosity after (min.).							
	1	2	3	4	6	10	14	20
Dialysed . . .	3·74	3·73	3·72	3·72	3·70	3·62	3·58	3·54
,, +0·002 M $CaCl_2$	3·22	3·03	2·82	2·66	2·50	2·14	1·98	1·83

In view of these results, it was of interest to see if a similar effect were produced with a pectic compound of different nature and having fewer carboxyl groups. A sample of pectin acetate was used containing 4 per cent. acetyl and 9·6 per cent. methoxyl groups. To 9 ml. of a one per cent. solution was added 1 ml. of a dialysed enzyme preparation or 1 ml. of the same preparation containing 0·02 M $CaCl_2$. The relative viscosities of the two mixtures over a 20-minute period are given in Table VIII.

As with pectin and sodium pectate, the addition of calcium gave a rapid reduction in viscosity which did not occur in its absence.

TABLE IX

Inactivation of Depolymerase by Heat

Treatment.	Relative activity	
	25% viscosity loss.	50% viscosity loss.
4 min. 25° C.	100	100
4 ,, 50° C.	55	47
4 ,, 60° C.	38	31
4 ,, 62·5° C.	21	18
4 ,, 65° C.	5	4
1 ,, ,, ...	65	51
2 ,, ,, ...	54	42
4 ,, ,, ...	28	23
6 ,, ,, ...	15	9
10 ,, ,, ...	5	9
20 ,, ,, ...	5	9

3. *Thermal inactivation.* In these tests a dialysed enzyme solution from an acetone precipitate was used. Aliquots of 2 ml. were placed in thin-walled glass tubes in water-baths at different temperatures. After a known exposure to these temperatures the tubes were transferred to ice-water where they were kept for a short period before adding to the substrate consisting of a 0·5 per cent. solution of pectin buffered at pH 8·3 in the presence of 0·002 M calcium chloride. The results for end-points representing a 25 or 50 per cent. loss of viscosity are given in Table IX. These low end-points were chosen to

obtain data for partially inactivated solutions which reduce the viscosity of the mixtures relatively slowly.

There was good agreement between the results obtained for the two end-points. Activity was reduced by some 50 per cent. after 4 minutes at 50° C.; at 62·5° C. loss of activity was very rapid and almost complete after 10 minutes' treatment. No data are included in this table for temperatures above 65° C. as the loss of activity was so great that it was impracticable to measure it over the 20-minute period used for these determinations.

4. *Inactivation at low pH values.* The procedure was essentially similar to that described in the last section. Aliquots of a partially purified preparation were brought to and maintained at different pH values with 0·01 N HCl for various periods. After making suitable corrections for the volumes of acid added the enzyme solutions were tested with 0·5 per cent. solutions of pectin buffered at pH 8·3 in 0·002 M CaCl$_2$. The results are shown in Table X.

TABLE X

Inactivation of Depolymerase at Low pH Values

Treatment.	Relative activity 50% viscosity loss.	Treatment.	Relative activity 50% viscosity loss.
Control maintained at pH 8·3 . . .	100	Control maintained at pH 8·3 . . .	100
1 min. at pH 2·7 .	47	15 min. at pH 3·7 .	55
5 ,, ,, . .	24	45 ,, ,, .	51
15 ,, ,, . .	11	195 ,, ,, .	33
45 ,, ,, . .	7	1,260 ,, ,, .	9
195 ,, ,, . .	2		

The enzyme was rapidly inactivated at pH 2·7, but was much less affected at pH 3·7. Storage overnight at pH 4·7 had little effect on activity. This rapid inactivation at a low pH is in marked contrast with the behaviour of fungal polygalacturonase, the latter being relatively sensitive to low pH values.

(*b*) *Protopectinase*

1. *Effect of pH on activity.* In these experiments aliquots of crude cell-free preparations were adjusted to various pH values with 0·1 N HCl or NaOH and brought to the same final volume. Six strips of tissue were placed in each solution and the reaction times determined; each strip was removed after maceration. The pH of the solutions was taken before the strips were placed in them and immediately after the last strip was removed. Except at the lowest pH there was a drop in pH of the solutions during maceration. Below pH 7·0 this was only slight, but above 7·0 the fall in pH became progressively greater until at the highest initial value, 10·4, a fall of 1·6 units was recorded. Better control of pH was obtained in the presence of veronal buffer

but, even so, there was a slight fall in the treatment having an initial pH of 10·2. Table XI gives data from one experiment; essentially similar results were obtained from others. The figures of pH are the means of the initial and final values of the solutions; activity is shown as 1000/reaction time in minutes.

TABLE XI

Effect of pH on Protopectinase Activity

pH.	Activity.
4·9	<5·5
5·9	9·7
6·2	17·5
6·6	23·3
7·0	25·0
8·2	29·4
8·6	40·0
9·1	41·6
9·6	52·6

There was no maceration within 6 hours in any of the controls in which autoclaved enzyme solutions were used. Activity was much reduced below pH 5·9 and increased rapidly up to pH 9·6. In this particular experiment no reversal in the trend of increasing activity with pH was obtained; in other experiments a slight drop in activity was recorded above pH 9·6. These results are in general agreement with those already described for depolymerase, particularly if the estimates of activity are considered in relation to the enzyme concentration and activity curve shown in Fig. 4.

2. *Effect of dialysis.* The activation of depolymerase by calcium ions in low concentration has already been noted. A similar, although not as striking an effect, has been obtained with protopectinase acting upon potato tissue. A 100-ml. sample of a crude cell-free preparation was dialysed in 2,000 ml. distilled water for 18 hours at 1° C. and then in 2,000-ml. volumes of distilled water for three 4-hour periods at 18° C. In each case the cellophane tube containing the enzyme solution was slowly rotated. The solution was then divided into two portions to one of which was added calcium chloride at a final concentration of 0·005 M. These and the undialysed preparations were adjusted to pH 8·3 with 0·1 N NaOH and water added where appropriate to bring the concentration of enzyme in each solution to the same value. The results of this experiment are given in Table XII.

TABLE XII

Effects of Dialysis and Calcium Chloride on Protopectinase Activity

Treatment.	Activity.
Undialysed	71·4
Dialysed	21·3
,, +0·005 M. $CaCl_2$	47·7

It is clear that the loss of activity following dialysis was partially restored on the addition of calcium chloride.

Similar effects were obtained when the calcium ions in enzyme solutions were removed with a cation exchange resin or with sodium oxalate.

3. *Thermal inactivation.* The procedure here was as already described for depolymerase. Reaction times were determined in the presence of veronal buffer at pH 8·3 and the estimates of activity from these results are shown in Table XIII.

TABLE XIII

Inactivation of Protopectinase by Heat

Treatment.	Activity.	Treatment.	Activity.
4 min. at 25° C.	58·5	1 min. at 62·5° C.	52·6
,, 50° C.	58·8	2 ,, ,,	40·0
,, 55° C.	47·7	3 ,, ,,	29·4
,, 60° C.	26·3	4 ,, ,,	22·7
,, 65° C.	10·6	6 ,, ,,	16·1
,, 70° C.	5·1	10 ,, ,,	10·5
,, 85° C.	4·2	20 ,, ,,	5·3
,, 100° C.	4·2		

The pattern of inactivation was substantially the same as that obtained for depolymerase. It is interesting to note that solutions retained some macerating activity even after treatment at 100° C. for 4 minutes.

TABLE XIV

Effect of pH on Inactivation of Protopectinase

Treatment.	Activity.	Treatment.	Activity.
Nil	45·4	15 min. at pH 3·7	30·3
1 min. at pH 2·7	21·7	45 ,, ,,	27·0
2 ,, ,,	17·2	105 ,, ,,	21·2
5 ,, ,,	14·9	195 ,, ,,	18·5
15 ,, ,,	13·7	21 hrs. ,,	7·8
45 ,, ,,	12·8		
75 ,, ,,	11·9		
105 ,, ,,	10·6		
195 ,, ,,	10·0		
270 ,, ,,	9·6		

4. *Inactivation at low pH values.* The methods described for depolymerase were again used. The results are summarized in Table XIV and are in general agreement with those for depolymerase.

Activity dropped rapidly on exposure at pH 2·7 but was much less affected at pH 3·7. Storage overnight at pH 4·7, however, produced a greater loss of protopectinase than of depolymerase activity.

5. *Effect of temperature on activity.* Solutions containing 25 per cent. by volume of a crude cell-free preparation and veronal buffer at pH 8·3 were maintained at the required temperatures in water baths and the reaction times determined in the usual way. Table XV shows the data obtained.

The results are not easy to interpret owing to the possible effects of temperature on processes not immediately connected with the initial breakdown of the insoluble pectic material of the tissue, e.g. high molecular weight compounds may be the first products of the reaction; an increase in temperature would increase the rate of diffusion of such substances away from the surface of the insoluble material and so facilitate the inward movement of enzyme molecules. The data illustrate, however, the considerable increase in activity with temperature, particularly if the figures representing activity are considered in the light of the relation between enzyme concentration and activity represented in Fig. 4, and indicate the desirability of working with fairly close temperature control, particularly over the range of 20–25° C.

TABLE XV

Effect of Temperature on Protopectinase Activity

Temperature at ° C.	Activity.
15	15·6
20	20·8
25	23·4
30	35·7
35	41·6
40	55·5

(*c*) *Chromatography of breakdown products.*

It has already been stated that prolonged treatment of solutions of pectin or sodium pectate with enzyme preparations resulted in increases of reducing power corresponding to approximately 20 and 40 per cent. respectively of the theoretical value for complete hydrolysis and that these figures are considerably below those reported for certain fungal polygalacturonases where galacturonic acid has been identified as the main end-product. It was therefore of interest to investigate the nature of the end-products produced by the action of the enzymes of *B. aroideae*. This was done chromatographically with Whatman No. 1 filter-paper and a solvent mixture consisting of butanol 45, ethanol 15, acetic acid 10, and water 35 parts by volume. The following were placed on the paper: 0·06 mg. and 0·006 mg. galacturonic acid, 0·04 ml. of 0·5 per cent. solutions of pectin or sodium pectate before addition of enzyme and after enzyme treatment for increasing periods over 7 days. The mixtures were incubated at 25° C. under toluene. As the main object of this work was to determine if and when galacturonic acid appeared in the reaction mixtures, no attempt was made to determine the R.F. values of the components which appeared. The chromatograms were developed for 4 days at 18–20° C., dried, sprayed with a solution of *m*-phenylene-diamine dihydrochloride, and heated for 5 minutes at 90° C. This reagent gave a characteristic pink colour with galacturonic acid, well-defined spots appearing at both concentrations. Similar spots were obtained when galacturonic acid in the same concentration was added to spots of the enzyme and substrate mixture or to this mixture 24 hours before placing on the filter-paper. Corresponding spots were absent in all other mixtures, but a well-defined spot, light brown in colour, regularly appeared above the galacturonic acid position. This was absent in the mixtures containing autoclaved enzyme as were other spots, somewhat less well defined, which appeared at a higher level. These results were confirmed with another solvent mixture containing butanol 40, acetic acid 10, and water 40 parts by volume and using benzidine and trichloracetic acid or *p*-anisidine hydrochloride as spraying reagents. These components which appeared on the chromatogram have not been identified; it may be noted, however, that although they are the end-products of the action of enzymes from *B. aroideae*, they may be further broken down to galacturonic acid by enzyme solutions derived from cultures of *Fusarium moniliforme*.

Discussion and Conclusions

A comparison will first be made between the enzyme provisionally described in this paper as depolymerase and the polygalacturonase type of enzyme which is obtained from fungal cultures or from commercial preparations such as are used in food technology. While a detailed comparison is not possible at this stage because the mode of action of depolymerase is still not known, it is clear that there are a number of important differences between the two enzymes. Thus the pH optima reported for polygalacturonase fall within the range 3·0–6·0; depolymerase when assayed viscometrically was virtually inactive at these values, but its activity increased rapidly above pH 7·0 and reached a maximum about pH 9·0. A further property distinguishing depolymerase from polygalacturonase is that it degraded solutions of high and low methoxyl pectins at similar rates. In contrast, polygalacturonase hydrolyses low methoxyl pectins much more rapidly than high methoxyl pectins, and in view of this it has been suggested that it acts only on the de-esterified portion of the polygalacturonide chain. In considering the action of depolymerase on high methoxyl pectins it must be remembered, however, that while the enzyme preparations containing it showed little or no pectinesterase activity, the rapid degradation occurred only at pH values at which alkaline de-esterification would take place if the period of incubation were sufficiently prolonged. But as the assays of activity were made at about pH 8·0 and were of short duration, generally 20 minutes, it is unlikely that de-esterification was a material factor in the degradation which occurred over this period.

Depolymerase also differs from polygalacturonase in being unstable under acid conditions. Thus at pH 2·7 some 90 per cent. of activity was lost in 15 minutes. In contrast, the relative stability of polygalacturonase in crude preparations at very low pH values has been used as a means of separating it from pectinesterase which is rapidly inactivated under these conditions.

Although activation of polygalacturonase by alkali salts has been reported, the effect is not as striking as that obtained with depolymerase by the addition of calcium. Moreover, the action of calcium seems to be specific and was not obtained with elements which are chemically very similar. Further study of the activation of depolymerase by calcium is obviously required, particularly in view of the possible importance of this element in building up insoluble frameworks in the cell-wall, and as a component of the middle lamella.

A further point of difference is that, even after prolonged incubation, the action of depolymerase on solutions of pectin or sodium pectate did not produce galacturonic acid. This is in marked contrast to the action of polygalacturonase where almost quantitative yields of galacturonic acid have been obtained from suitable substrates. Solutions degraded by depolymerase contained a number of end-products of which at least one was of fairly low molecular weight and was possibly a digalacturonide; these end-products only appeared in substantial quantities after relatively long incubation.

Finally, in contrast to many pectic enzymes of fungal origin, including polygalacturonase, the production of depolymerase was non-adaptive in the sense that it was independent of the presence of pectic substances in the culture medium. The effect of such substances on the production of depolymerase and other pectic enzymes by *B. aroideae* will be dealt with in a later paper.

It is clear therefore that depolymerase cannot be identified with polygalacturonase of fungal origin. It does, however, resemble in some ways certain other pectic enzymes which have been recently described and which also differ in their mode of action and properties from polygalacturonase. Even with these there are important points of difference. Thus, it may be distinguished from the depolymerases of tomato fruit (McColloch and Kertesz, 1948), of yeasts (Luh and Phaff, 1951), and *Neurospora crassa* (Roboz et al., 1952) and the polymethylgalacturonase obtained from a commercial product (Seegmiller and Jansen, 1952) in having a much higher pH optimum, and further differs from tomato fruit depolymerase and polymethylgalacturonase in degrading solutions of soluble pectate and pectin at approximately equal rates. At present it would seem justified to consider the viscosity-reducing enzyme secreted by *B. aroideae* as a distinct enzyme and to call it a *bacterial depolymerase*.

The identity of this enzyme with the enzyme in the bacterial preparations which liberated reducing groups from solutions of sodium pectate and pectin must remain conjectural until more is known of the properties of the second enzyme. It may be noted, however, that the enzyme preparations used in this work were more active, in terms of reducing groups liberated, with solutions of pectate than with pectin solutions as substrates. In this respect they resemble preparations of polygalacturonase, although the effect of the two substrates is not as striking. It is, of course, quite possible that an enzyme resembling polygalacturonase in its mode of action is present in the bacterial culture fluids together with a depolymerase. If this were so, then it would be envisaged that the latter enzyme acted upon linkages in the polygalacturonide chain which, on rupture, did *not* yield reducing groups. Evidence for such linkages is increasing; pectic substances are known to contain a proportion of non-uronide material which may be as high as 10 per cent. in the case of citrus pectin. If such material forms an integral part of the chain molecule and is distributed fairly uniformly along its length, then an enzyme acting upon linkages between uronide and non-material would bring about a very rapid reduction in viscosity and might, at the same time, not liberate reducing groups. The action of polygalacturonase might be considerably slower and would be confined to the uronide fractions after these had been de-esterified either by pectin-esterase or, in the case of *B. aroideae*, by the relatively high pH values required for good activity.

It now remains to consider the relation between the enzymes discussed above and protopectinase. So far the latter has been demonstrated only by its action on plant tissues, and a chemical description of the reactions leading to maceration will not be possible, even in approximate terms, until more is known of the nature of protopectin itself. There is, too, the possibility that pectic enzymes are not solely responsible for maceration; other enzymes acting upon the non-pectic components of the cell-wall may well be involved. In this connexion, however, it may be mentioned that bacterial preparations which rapidly macerate plant tissues have little if any cellulose activity when tested on solutions of sodium carboxymethylcellulose.

Protopectinase has not yet been identified as a distinct enzyme; in recent years there has been a growing tendency to equate its activity with that of a polygalacturonase acting in combination with pectin-esterase. This view is supported by the fact that protopectinase preparations always contain enzymes having polygalacturonase or depolymerase activity. The bacterial preparations used in the work described above behaved in this manner even after partial purification in a number of ways. The properties of the depolymerase and protopectinase in these preparations were similar. Thus, they responded in the same general way to pH, inactivation by low pH values and heat, dialysis and precipitation by alcohol, acetone, or ammonium sulphate. A striking similarity was the way in which both were activated by calcium. Although this effect was less pronounced for protopectinase, this might be expected in view of the probability that calcium was released from the test tissue during maceration. As a further example of the similar behaviour of the two enzymes, the following may be quoted. In testing the ability of enzyme preparations to macerate a variety of tissues it was noticed that red-beetroot tissue was particularly resistant, e.g. slices 0·5 mm. thick were macerated only after 5–12 hours' treatment compared with 10–15 minutes for slices of potato tuber. A pectin was prepared from red-beetroot in the usual way; samples of the enzyme solution used in maceration tests reduced the viscosity of 0·5 per cent. solutions of this pectin only very slowly compared with solutions of 0·5 per cent. citrus pectin.

Although there was a general agreement in the behaviour of the two enzymes there were some differences in detail. These were most pronounced at different pH values. Both enzymes had an optimum close to pH 9·0. Below this value, however, depolymerase activity decreased more rapidly than protopectinase activity so that at pH 6·0–7·0 there was a divergence between the activities of the two enzymes not apparent at higher values. Further study of this and other differences is obviously required before the identity of the enzyme or group of enzymes responsible for maceration can be established.

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Investigations on the Growth and Metabolism of Plant Cells

V. Tumorous Growth in Relation to Growth Factors of the Type found in Coconut

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With Plate I and three Figures in the Text

ABSTRACT

1. It has been shown that aqueous extracts of plant tumours, induced by *Agrobacterium tumefaciens* (Smith and Townsend) on *Bryophyllum* and *Kalanchöe*, will act in place of coconut milk using the tissue-culture procedures previously described in this series of papers.

2. In a large number of experiments it has been shown that tumours of this kind yield extracts which have a growth-promoting effect similar to that of coconut milk. This effect may be enhanced by, though it is distinguishable from the effects of, added casein hydrolysate in the basal medium. The activity of the tumour extracts was consistently greater than the activity of extracts of stems and leaves of the same plants and of normal, non-tumorous plants.

3. Partial fractionation of the tumour extracts has shown that activity was concentrated in the alcohol extracts, and to a lesser extent in the water extracts. Activity was completely lacking in the ether extracts.

4. The effect of coconut milk, which is replaceable wholly or in part by tumour extracts, is primarily an effect on cell division in the carrot tissue.

5. The bearing of these results on tumorization in plants is discussed.

INTRODUCTION

THE discovery that coconut milk (the liquid endosperm of *Cocos nucifera*) could induce rapid proliferative growth in certain mature tissues explanted from the plant body raises the question of the possible relationship of the substances it contains to tumorous growth. The carrot-root explants, consisting of secondary phloem (Pl. I (i)), grow rapidly when supplied with a complete nutrient medium supplemented with coconut milk, and they will continue to grow indefinitely in the presence of the coconut milk on repeated subculture (unpubl.). The general nature of this proliferative growth in these

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cultured tissues, initiated in cells that would not, in the carrot root, normally have divided again even though they will expand in the basal medium (Pl. I (vi)) is suggestive of the growth of plant tumours (Pl. I (vii)).

Many roots furnish explants which virtually do not grow at all in the basal medium under the conditions of these experiments; the example cited in Pl. I showed a relatively large increment. As the plate shows, this particular stock of carrot was able to gain in weight quite appreciably in the basal medium. Within the period of observation this increment was mainly enlargement of the smaller cells in the original explant. Different carrot roots vary markedly in the ability of their phloem explants to grow in the basal medium. In the presence of the coconut milk a great stimulus to cell division is invariably produced. A curious but obviously erroneous idea was suggested by White (1953), namely, that the relatively large increments in growth produced by coconut milk are related to cell enlargement (loc. cit., p. 522) rather than to cell division. From such evidence as that in Pl. I, this is clearly not so. A 2-mg. carrot explant may originally have about 25,000 cells. After about 14 to 20 days of growth under the conditions prescribed the number may be of the order of 1,000,000! The very rapid growth of the culture causes air spaces to appear in the central mass. On the average, the cell size of cultured carrot tissue remains small, even smaller than in the original tissue, though some large cells occur towards the periphery (Pl. I (vii)). The presence of air spaces and the curious scalariform elements, which are also encountered in certain tumours, were both highly characteristic of this cultured tissue.

The agents in the coconut milk which cause the otherwise mature carrot cells to grow so rapidly may, therefore, shed light upon the stimuli responsible for the growth of the tumours. This problem has been explored by particular reference to the tumours that can be initiated and grown on *Bryophyllum*, *Kalanchöe*, and tomato (*Lycopersicon*) by the use of the crown-gall organism (*Agrobacterium tumefaciens*) (Smith and Townsend, Bergey et al.). The bacterial cultures for use in this work were obtained from Dr. A. J. Riker of the University of Wisconsin.

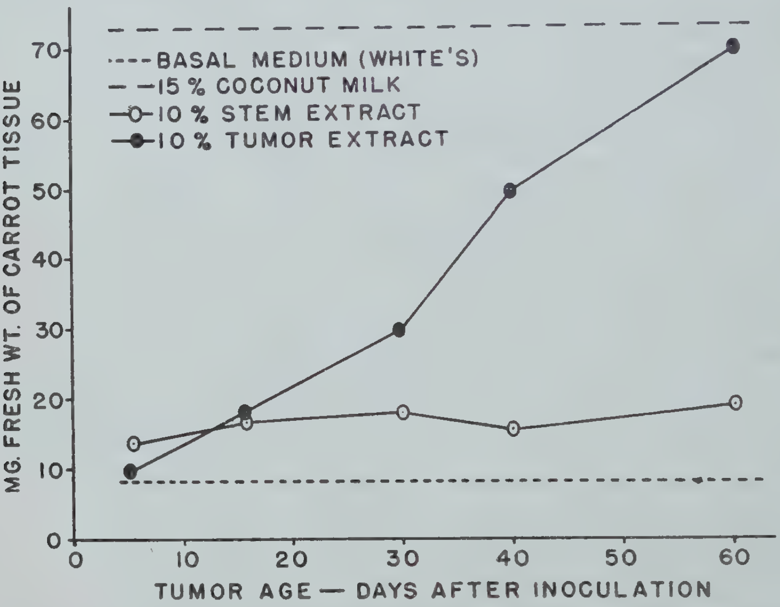
In Parts III and IV of this series (Steward and Caplin, 1952 *a* and *b*) note has been taken of the fact that most mature cells of the plant body fail to respond to the nutrient media commonly employed for the growth of tissue cultures for the following reasons. In the first place, the tissue may require external supplies of the growth factors which are contained in the coconut milk. In the second place, the tissue may be unable to respond to such accessory growth factors even when they are supplied because it may contain inhibitory substances or mechanisms. This being so, there are two distinct ways in which an external agent (bacteria or other agent) may incite otherwise mature cells to proliferate, as in a tumour. The external agent could supply the missing growth factors, or it could induce the cells to become autonomous for them, whereby a true tumorization of the cells would occur. Alternatively, the external agent could antagonize, or otherwise neutralize, the controlling effect of inhibiting factors or mechanisms for growth that the cells contain or could produce. (The suggestion has been made (Steward and Caplin, 1952*a*) that the role of 2,4-D in stimulating the cells of the potato to grow (Steward and Caplin, 1951) may be of this latter kind.) Similar considerations might apply in those cases in which tumours arise in plants spontaneously, as, for example, in the well-known effect of genetic constitution which determines spontaneous tumour formation in *Nicotiana* hybrids (Kehr and Smith, 1952).

It had already been shown by DeRopp (1947) that bacteria-free crown-gall tissue grown on agar generates a diffusible substance, or substances, which induces proliferation in callus tissue, suggesting that growth-promoting substances similar to those occurring in coconut milk might be produced autonomously by cells stimulated into rapid growth by the crown-gall organism. Conversely, Duhamet (1951) has recently shown that the growth of various types of crown-gall tissue, such as those of grapevine, tobacco, salsify, and Jerusalem artichoke, may be markedly increased in the presence of coconut milk. There is therefore some evidence that a principle promoting cell division may be formed by crown-gall tissue and, conversely, the crown-gall tissue in culture can respond to the substances in coconut milk that are now known to promote cell division.

In previous papers use has been made of the carrot-coconut milk assay procedure (Steward, Caplin, and Millar, 1952; Caplin and Steward, 1952) to detect the presence of active growth factors equivalent in their action to coconut milk itself (Steward and Caplin, 1952*b*), or the presence of growth-inhibiting substances (Steward and Caplin, 1952*a*). In application to the tumour problem the methods permit the detection of growth factors for carrot tissue if they are produced by the tumorous cells. If, however, tumorous growth is due to the inactivation of an inhibitor normally present in the non-tumorous cells, the method can be used to detect this also.

It is now known, however, that the over-all effect hitherto described as 'Coconut Milk Growth Factor' is itself due to several distinct substances. The growth-promoting effect of coconut milk on carrot tissue is divisible into two distinct parts. For one of these coconut milk is not a unique source, for it may be supplied by the use of more general nitrogenous sources, such as casein hydrolysate and amino-acid mixtures. The other, for which coconut milk, or the comparable sources mentioned previously (Steward and Caplin, 1952*b*, II) is a much more specific source, is due to a group of substances that, although as yet not completely identified, have been obtained in pure crystalline form (compounds A, B, and C of Shantz and Steward, 1952). The respective requirements of carrot phloem explants for these two classes of growth-promoting substances are complex and they will be described in much more detail in another paper. However, it should be mentioned here that carrots from some stocks yield explants that are much less responsive to a casein hydrolysate supplement to the basal medium than are others. Invariably, however, the addition of the more specific growth factors, whether in purified extracts or in whole coconut milk (Pl. I, *cf* (iii) and (iv)), stimulates growth far in excess of that due to casein hydrolysate alone (and also much more than any response obtainable by the use of indoleacetic acid). Therefore, in testing extracts of plant tumours for growth-promoting effects of the kind hitherto called 'coconut milk factor' the tests have been made both with and without casein hydrolysate in order to be sure that a negative result would not arise for lack of the less specific constituents of the casein hydrolysate.

Adopting this line of thought, the following type of experiment was carried out. Tumours were grown on *Kalanchöe* stem by the standard needle puncture method using *Agrobacterium tumefaciens.* The tumours were allowed to grow for periods varying from 1 to 3 months after inoculation. The tumours were then harvested and extracted with hot water (1:1). These aqueous extracts were tested simultaneously by using them at various concentrations in the place of coconut milk in the carrot assay procedure. The first results obtained are shown in Text-fig. 1.[1] From this it seemed that as the tumours grew they became progressively richer in constituents that were able to support the growth of carrot in the manner normally obtained with coconut milk. This result is consistent with the presence in these tumours of the specific growth factors of coconut milk, or their equivalent, and their continued autonomous production in the tumour as it grows. Both the mature stems of *Kalanchöe* and the cultured bacterium were also tested by the carrot assay



TEXT-FIG. 1. Growth of carrot extracts in 14 days on media containing extracts from normal stem of *Kalanchöe* and from stem tumors induced by *A. Tumefaciens.*

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[1] At this point the writers wish to acknowledge that these preliminary experiments were carried out by Dr. Richard Klein, then working in the Biological Laboratories at Rochester under a National Institutes of Health Predoctoral Fellowship under the direction of one of us (F. C. S.). It was from this starting-point that the subsequent investigation developed.

method. Although some activity was obtained with the extracted cells of the bacterium, this did not reproduce the effect of the whole coconut milk as did the extracts of the tumours.

There was, therefore, preliminary evidence to support the view that the stimulus to growth in *Bryophyllum* or *Kalanchöe* tumours may be similar to the chemical stimuli that the coconut milk furnishes to the carrot explants, for water extracts of the tumour can act in the place of coconut milk and induce the growth of carrot explants. It is true that the ability of aqueous extracts of tumour to support the growth of carrot explants has not invariably proved to be so direct and regular a function (as in Text-fig. 1) of the age of the tumour from which the extract was obtained. Nevertheless, the *fact* that this activity occurs in the tumours seems to be abundantly demonstrated, as will now be shown.

THE EFFECT OF EXTRACTS OF TUMOURS ON GROWTH OF STANDARD CARROT EXPLANTS COMPARED WITH THE EFFECT OF COCONUT MILK

The following series of experiments yielded positive results when extracts of tumour tissue were tested by the carrot assay method, i.e. the tumour extract could act in some degree in the place of coconut milk. The experiments which yielded these positive results are presented in sequence, for they show that the cumulative evidence is strong and that positive results have been obtained over a long period and with various stocks of carrots as the assay material.[1]

Expt. 75. In this experiment the growth (increase in fresh weight) of carrot explants in a medium containing an aqueous extract of *Kalanchöe* tumours was 85 per cent. of that obtained with an optimum concentration of coconut milk. Extracts of tumours on tomato and marigold, tested concurrently on the same population of carrot explants, were less active and gave an increase corresponding to 58 and 25 per cent. respectively, of the increase in fresh weight of the controls on a complete medium (basal medium plus coconut milk).

Expt. 308. Here comparisons were drawn between the growth-promoting activity of extracts of the tumour in comparison with similar extracts from leaf and stem tissues. The extract added to the basal medium (Caplin and Steward, 1952) corresponded to 0·1 g. of fresh tissue per 10 ml. of the medium, which contained one carrot explant.

As shown by Text-fig. 2, the aqueous extract of the vegetative tissue of the *Bryophyllum* plant had only small growth-promoting activity, while the extract of the tumours had greater growth-promoting power for carrot explants than the extract of the stem on which the tumours were borne, and had greater activity than the leaves of the same plant. The tumour extract gave over 50 per cent. of the growth which was stimulated by coconut milk.

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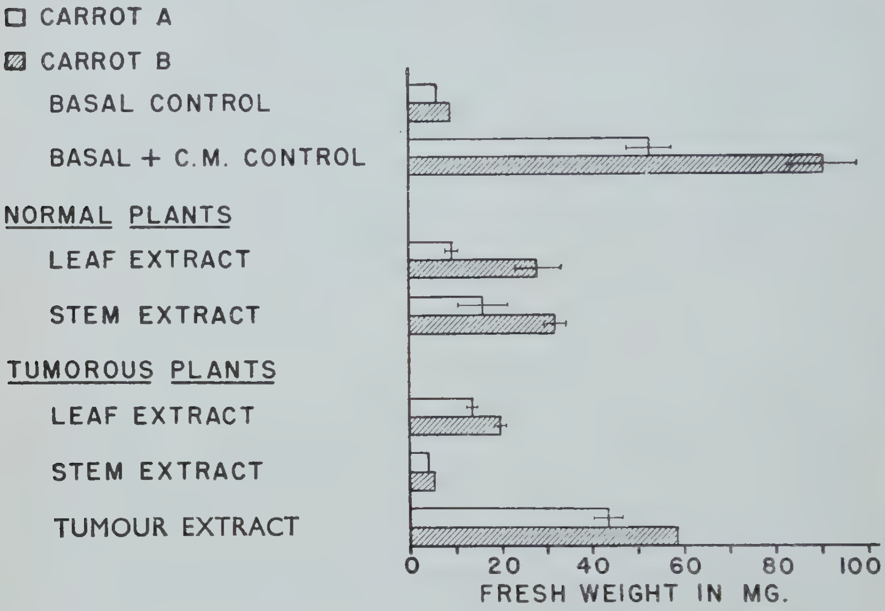
[1] The experiments referred to by number refer to a long series of tissue culture experiments mainly carried out at the University of Rochester.

Expt. 316. In this experiment tumours which had grown for 8 months on *Bryophyllum* were extracted and tested using standard carrot explants (3 mg.) which were allowed to grow for 14 days in 10 ml. of liquid medium under the controlled conditions of these experiments. Various concentrations of the tumour extract were added to the basal medium in place of coconut milk.



TEXT-FIG. 2. Effect of *Bryophyllum* extracts on growth of carrot explants.

As shown by Text-fig. 3, there was a progressively greater growth with concentration of the tumour extract and at the highest concentration tested (4 per cent.) the tumour extract replaced the effect of 15 per cent. coconut milk in the medium to the extent of between 80 and 100 per cent. (depending on the individual carrot from which the explants were removed).

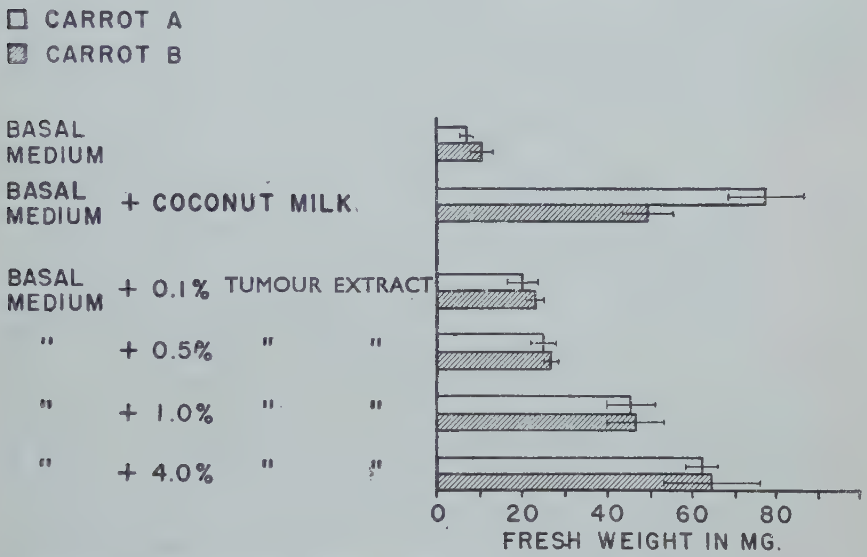
TABLE I

Combined Effect of Coconut Milk and Tumour Extract on Growth (mgm. Fresh Wt.) of Carrot Explants

Carrot root	% of tumour extract to basal medium+3% CM				
	0	0·1	0·5	1·0	4·0
‘A’	85·1±7·5	95·0±3·6	—	74·1±3·1	86·1±6·0
‘B’	34·4±2·2	54·5±2·2	44·8±3·4	35·7±6·2	37·0±2·7

In this experiment explants from both carrots grew well with addition of 3 per cent. coconut milk, by volume, and addition of a further 0·1 per cent. of tumour extract caused even further increase in growth. At still higher concentrations of tumour extract this additional stimulus was progressively reduced (Table I, see especially carrot ‘B’).

These data suggest, therefore, that addition of tumour extract is very similar to addition of coconut milk itself, the optimum concentration for these samples being at a total concentration of less than 15 per cent. by volume of coconut milk. At a concentration of 3 per cent. coconut milk plus a low concentration (0·1 per cent.) of tumour extract, the growth-promoting qualities for carrot explants were increased; at higher concentrations of added tumour extract the optimal activity was exceeded and the total growth was, in consequence, reduced.



TEXT-FIG. 3. Effect of *Bryophyllum* tumour extracts on growth of carrot extracts.

All of these experiments are compatible with the idea that an extract of tumour tissue contains growth-promoting qualities for carrot explants in excess of that commonly found in the mature tissues of the plant body. Not only does this growth-promoting quality replace coconut milk in large part, but it can supplement it when the latter is furnished to carrot explants in suboptimal concentrations.

Expt. 330. In this experiment aqueous extracts from tumours borne on various plants were tested at a concentration of 1 per cent. (equivalent of 0·1 g. of tissue per 10 ml. of culture medium) for their ability to induce growth of carrot explants when added to the basal medium in place of, or in addition to, coconut milk. The data are shown in Table II.

In most of these cases the tumour extract supplemented the basal medium and stimulated a large part of the growth of carrot explants that otherwise occurred in the presence of 1 per cent. coconut milk. Only in the case of *Bryophyllum* tumour was there any evidence that the tumour extracts *increased* the growth-promoting qualities of a dilute coconut milk preparation (even this effect was only significant for carrot 'B'). In all of the other cases the addition of a tumour extract to a medium containing coconut milk did not *increase* the effectiveness of the latter though, in fact, towards one carrot, 'A', they seemed significantly to reduce the growth of the carrot explants as though they might have caused some form of inhibition. However, the interesting feature is that the growth on the media which contained an extract of tumour tissue far exceeded the growth on the *basal* medium, and it corresponded at 1 per cent. to a large part of the activity that could be obtained with 1 per cent. coconut milk. Positive growth-promoting responses for carrot explants, of the kind normally obtainable with coconut milk, were thus detected in the extracts from tumours grown on *Bryophyllum*, on African marigold (*Tagetes erecta*), *Cosmos*, and *Coleus*, but *not* in the tumours on French marigold (*Tagetes patula*) or on sunflower (*Helianthus annuus*). (It may be mentioned here that the most consistently positive growth-promoting effects were obtained by applying extracts of *Bryophyllum* or *Kalanchöe* tumours to carrot explants.)

Table II

Effect in 14 Days at a Concentration of 1 per cent. of Extracts of Various Tumours on Growth of 3 mg. Explants of Carrot Root in Basal Medium containing Coconut Milk*

Carrot.		Basal medium.	*Bryophyllum* tumour.	African marigold tumour.	*Cosmos* tumour.	*Coleus* tumour.	French marigold tumour.	Sunflower tumour.
A	No CM	17·3±8·2	57·8±10·7	57·0±24·3	41·4±9·3	33·4± 5·3	23·1± 1·6	14·0±1·2
A	+1% CM	77·1±1·3	86·6±18·7	55·9± 2·0	53·1±9·6	60·7±12·0	43·0±11·7	46·6±9·7
B	No CM	11·1±1·9	50·3± 3·5	30·8± 4·2	29·3±1·3	24·2± 1·4	7·8± 1·8	11·8±3·7
B	+1% CM	43·6±2·7	72·2± 5·7	47·2± 6·7	44·0±7·4	47·6± 9·1	22·0± 7·2	43·0±7·1

* 1% = the equivalent of 0·1 g. of tumour in 10 ml. of medium.

Expt. 413. Since the growth-promoting activity of coconut milk for carrot explants is divisible into a fraction replaceable by casein hydrolysate at very low concentrations (for which coconut milk itself may not always be a fully adequate source for all kinds of carrot cells) and a group of other factors (for which coconut milk or similar material is a more specific source), an attempt was made to determine in which of these two categories the activity of the tumour extract might be found. The tumours were grown on *Kalanchöe* stems for 1 month. Hot water extracts were made of the tumours, stems, and leaves of the tumour-bearing plants and similar extracts were made of normal plants not bearing tumours. These extracts were added in place of coconut milk to the basal medium for carrot explants to which was added 0·05 per cent. casein hydrolysate.

The tissue extracts were tested for their ability to promote growth of carrot explants at a range of concentrations, namely 1, 5, 10, and 20 per cent., where 1 per cent. represents an amount of extract equivalent to 1 g. of tissue added to 100 ml. of the nutrient medium. The data will not be presented in full, but the following points are relevant.

The particular batch of carrot explants used in this assay responded only slightly when casein hydrolysate was added to the basal medium, and the increased growth due to casein hydrolysate when combined with coconut milk and the basal medium was also not significant. These data are shown in Table III.

The full growth of these explants during the assay period was, therefore, represented by the increase from 3 mg. to 120 mg. in basal medium supplemented by coconut milk plus casein hydrolysate. In large part this could be reproduced by the use of 0·05 per cent. casein hydrolysate combined with tumour extract and, at the optimum concentration of the latter (5 per cent.), the explants grew to 73·4±4·2 mg. Thus the combination of tumour extract plus casein hydrolysate reproduced over 50 per cent. of the growth-promoting effect of coconut milk plus casein hydrolysate. The tumour extract alone, i.e. lacking casein hydrolysate, was ineffective and gave only 18·5±3·7 mg. at its most effective concentration (1 per cent.). However, granted the addition of casein hydrolysate to the basal medium used in the assay, growth-promoting activity was found in other parts of the tumour-bearing plant, though it seemed not to be as great as the activity of the tumour extract even at the most effective concentration (10 per cent.). (The extract of leaves of the tumour-bearing plant gave 59·9±9·2 mg. and the stems gave 63·1±6·7 mg. of carrot growth.) Extracts of leaves and stems of normal (non-tumour bearing) plants also showed some growth-promoting activity when added to basal medium plus 0·05 per cent. casein hydrolysate, but the growth stimulated on the carrot explants was less than that caused by the extracts of the tumorous plant even at the most effective concentration (10 per cent.). (The leaf extracts from non-tumorous plants gave 47·6±8·3 mg. and the stems 46·2±10·5 mg. of carrot growth.)

TABLE III

Growth of 3 mg. Carrot Explants in Media with and without Casein Hydrolysate

		Plus casein hydrolysate	
Basal medium.	Basal+C.M.	Basal medium.	Basal+C.M.
5·1±0·2	103·9±10·1	18·2±2·4	119·7±22·5

This kind of data seems to mean that the *Kalanchöe* plant *lacks, or is deficient in, that part of the growth-promoting requirements for carrot explants which is to be found in casein hydrolysate.* If this is supplied, leaf and stem extracts of this plant, which readily form tumours, show appreciable ability to stimulate growth of carrot explants in ways that are otherwise only possible by the use of coconut milk, or extracts from somewhat similar morphological materials (Steward and Caplin, 1952). However, the presence of tumours increases the activity of the leaves and stems *in that moiety of the requirement of carrot explants for which coconut milk is the source and the tumour itself is an even more effective source than the rest of the plant body of the tumorous plant.*

These data would be coherent if it were concluded that:

(*a*) *Kalanchöe* contains and can form the specific coconut milk growth factors or equivalent substances.
(*b*) *Kalanchöe* is deficient in the casein hydrolysate moiety of the complex of growth factors required by carrot explants.
(*c*) The stimulation of growth in the formation of the tumour results in a greater and localized activity comparable to the coconut milk growth factors for carrot tissue.

Expt. 414. In this experiment the ability of an extract of *Kalanchöe* tumours to support the growth of carrot explants in presence of casein hydrolysate was confirmed, as well as the great growth-promoting activity of the tumour extract compared with a leaf extract. The tumours were grown on *Kalanchöe* for 2 months. The tests were made on minced, or homogenized, tissue, added to the nutrient medium in lieu of coconut milk. The assays were made on two populations of explants derived from different carrots (A and B) from the same stock. These responded in 19 days to the different nutrient media in the manner described in Table IV.

TABLE IV

Growth in mg. Fresh Wt. of Carrot Explants (3 mg.) in Media with and without Casein Hydrolysate

			Plus casein hydrolysate	
Carrot.	Basal medium.	Basal medium +C.M.	Basal medium.	Basal medium +C.M.
A	6·8±0·26	143·6±42·7	24·3±4·8	159·4±14·0
B	11·6±0·12	122·2± 8·7	17·6±5·6	101·6±35·5

Again these carrot explants show some slight response to casein hydrolysate in the basal medium, this effect being either additive to (carrot A) or submerged in (carrot B), the effect of whole coconut milk.

At the most effective concentration tested (10 per cent. = extract from 10 g. tissue included in 100 ml. of medium), the comparison of the tumour and other tissues gave the following data (Table V). The tests were made by growing carrot explants on the basal medium plus casein hydrolysate and by adding the tissue extract in the place of coconut milk.

It is immediately obvious that the tumour extract supplied some growth-promoting quality which acted in the presence of casein hydrolysate and which supplemented the basal medium to the extent that it now supported growth up to about two-thirds of that obtained with whole coconut milk; and, it will be noted, this result is true even though different carrot roots supply explants with somewhat different potentialities for growth. Again it is quite clear that the growth of the tumour tissue is associated with the increased production of some specific growth factor, or factors, which require for their action substances present in casein hydrolysate and which, like coconut milk, stimulate the growth of carrot explants. These factors, though possibly present in the mature tissue of *Kalanchöe* in residual amounts, are much more active in extracts of the tumour tissue.

TABLE V

Growth of Carrot Explants in mg. Fresh Wt. in Media containing Extracts of Plants with and without Tumours

	Extracts from plants bearing tumours		Extracts from normal plants
Carrot.	+Tumour ext. at 10%	+Leaf ext. at 10%	+Leaf ext. at 10%
A	104·5±15·8	54·3±15·2	51·2±7·4
B	80·8± 6·4	38·4± 7·5	35·9±6·4

Data from a further experiment (Expt. 423) again confirm the idea that the tumours borne on *Kalanchöe* plants yield extracts which act in lieu of coconut milk to support growth of carrot explants. Tumours 2 months old yielded the following data when tested against carrot explants for 15 days—the data are given only for the concentration of extract which produced the greatest growth.

TABLE VI

Growth of 3 mg. Carrot Explants during 10 days in Various Media showing the Effect of Extracts of Kalanchöe

Basal medium+casein hydrolysate	+C.M.	Basal medium+casein hydrolysate +extract of *Kalanchöe*		
		Tumour-bearing plants		Normal plants. Leaves.
		Tumours.	Leaves.	
21·8±5·0	99·4±9·0	83·8±10·9	24·7±7·9	54·1±0·5

The cumulative effect of the foregoing experiments is strong and points to a difference in the growth-promoting substances that are contained in plant tumours and in the other parts of the normal plant body. The results of the carrot assays indicate that the tumours are richer in substances that, when extracted, can stimulate carrot phloem cells to grow. In their effect upon carrot cells these substances are not replaceable by small amounts of casein hydrolysate, although they may only exert their effect when growth is not limited by lack of substances that are present in casein hydrolysate. The substances in question appear to be similar to, if they are not identical with, those that exist in coconut milk and which seem specifically to induce cell division in mature carrot tissue. These substances, or others that have homologous action, are now known to exist not only in the liquid endosperm of the coconut but in other comparable morphological situations in which the food storage for an embryo develops precociously, while the embryo remains immature (Steward and Caplin, 1952*b*). It is therefore of great interest that growing sterile plant tumours, which grow by random cell proliferation, may also contain these growth-promoting substances.

It should be recognized that the data quoted are typical of those in which positive results are obtained. Generally speaking, tumours formed on *Bryophyllum* or on *Kalanchöe* gave more uniformly positive results than those on other plants. Although some positive responses were obtained with the genetically determined tumours which form on the tobacco hydrid *N. glauca*×*N. langsdorfii*, experiments on these plants have not led, as yet, to clear-cut differences between extracts of the hybrid and of the parent plants which would be indicative of a marked tendency of the hybrid to form growth-promoting substances, assayable by the carrot tissue culture method. Similarly, tests have been made of a *Nicotiana* amphidiploid which spontaneously forms tumours (Kehr and Smith, 1952) and which has been regarded as doing this because of 'hormonal imbalance' as a consequence of its genetic constitution. It must also be admitted that tests made on these latter systems have not, as yet, yielded data which were decisive. This may not be wholly surprising since the method of hot water extraction is somewhat crude and the methods of extraction and isolation of pure factors are too laborious to be applied to small amounts of tumour material (Shantz and Steward, 1952). Also, this group of growth factors may well be only one part of a more complex situation leading to growth in tumours.

At this point, more positive and direct evidence was sought by fractioning extracts of the *Kalanchöe* or *Bryophyllum* tumours in order to show that substances with activity comparable to coconut milk fractions can actually be obtained from the tumours in greater quantity than from the leaves and stems of the same plants.

Partial Fractionation of Extracts from Tumours and their Activity on the Carrot Assay

The isolation from coconut milk of chemically pure growth substances, which incite cell division in carrot cells, presents a very formidable problem requiring a large amount of material. Our purpose was not to emulate this on plant tumour material, however interesting this might have been. The plan was, instead, to make extracts in a more selective way and from a larger amount of tumour tissue than was used in the experiments described earlier and, by concentrating these somewhat, gain unequivocal evidence of the existence in the tumour of active substances comparable to those that occur in coconut milk.

The method adopted was as follows.

Tumours were grown on the stems of *Bryophyllum*. Some of these were allowed to reach rather large size and these are described as 'Old tumours' the average weight of which was 55 g. Other tumours were harvested when they were younger and are described as 'Young tumours', the average weight of which was 1·7 g. For comparison, normal stem and leaf tissue of similar plants was harvested and subjected to the same treatment throughout.

Extracts of old and young tumours, and leaves and stems of normal plants, were made in the following way and will be designated Ether Extract, Alcohol Extract, and Water Extract, respectively. The fresh tissues were minced in a Waring blendor with five times their weight of absolute ethanol and allowed to stand overnight. The mixture was filtered and the residue was boiled in a second quantity of absolute ethanol equivalent to the first extract. After filtration the alcohol extracts were combined and evaporated to dryness under reduced pressure. The alcohol-extractable material was dissolved in water and the aqueous solution was then extracted three times with ethyl ether. The combined ether extracts were evaporated to dryness, weighed, and redissolved in a measured volume of ether. To make aqueous solutions for bioassay, since the ether-extracted material was but slightly soluble in water, a suitable aliquot of the ether solution was added to boiling water to remove the solvent.

The portion of the alcohol-extracted material which remained in the aqueous phase upon extraction with ether was dried, weighed, and redissolved in a measured volume of water.

The residual tissue, which had been twice extracted with absolute ethanol, was then extracted with boiling water. After filtering, the water extract was concentrated to a small measured volume, of which an aliquot was dried and weighed.

The amount of material obtained in each of the three extracts for each of the four tissue samples, expressed as mg. per gramme of fresh weight, was as follows:

TABLE VII

	Young tumours.	Old tumours.	Leaves.	Stems.
Ether extract . .	3·8 mg./g.	2·5 mg./g.	9·5 mg./g.	2·7 mg./g.
Alcohol extract . .	10·0	14·2	19·8	6·7
Water extract . .	9·3	9·7	37·7	10·2

The alcohol and water extracts showed optimal activity in the range of 400–800 p.p.m. in the nutrient medium. The ether extracts, which were inhibitory at these concentrations, were tested in the range of 2–20 p.p.m. in the culture medium.

Having obtained these extracts, the assay procedure was as follows: In each experiment (1 to 6 of Table VIII) assays were carried out with populations of explants derived from two carrot roots designated (A) and (B). Though the roots were from the same stock, this procedure takes some account of their individual idiosyncrasies. Furthermore, all assays were repeated to determine the effect of the extract when added to the basal nutrient medium, i.e. in place of coconut milk in the usual carrot culture method, and also when added to the basal medium supplemented by casein hydrolysate. Each entry in Table VIII was derived from three experimental observations, made on quadruple cultures. These treatments—in the absence of casein hydrolysate—were: the growth of explants in the basal medium, the growth of explants in the basal medium plus coconut milk, and the growth of explants in the basal medium plus tumour extract. The same comparisons were also made with added casein hydrolysate. The data in the table are first arranged according to the type of extract tested (series I–III), although the observations thus compared derive from five different experiments. Finally, comparisons are made (series IV) between alcohol extract from the four different kinds of plant material on assays which are made in a single experiment (expt. 6).

TABLE VIII

Growth-promoting Activity for Carrot Tissue of Extracts from Bryophyllum (Comparing Tumours and Normal Tissue)

Code: *a* Young tumours
b Old tumours
c Normal stems
d Normal leaves

(i) Ether extract
(ii) Alcohol extract
(iii) Water extract

(A) and (B) denote the particular carrots from which explants were taken.

Effect of extracts on growth expressed as:

$$\frac{\text{Growth increment due to extract}}{\text{Growth increment due to coconut milk}} \times 100$$

			Assays in absence of casein hydrolysate		Assays in presence of casein hydrolysate	
Series.	Expt. No.	Ext. added.	(A).	(B).	(A).	(B).
I	1	*a*–(i)	11·1	5·1	8·0	17·1
	2	*b*–(i)	4·6	−0·6	2·4	16·8
	3	*c*–(i)	1·3	−0·7	3·0	−0·6
	3	*d*–(i)	1·8	−3·2	0·9	−1·8
II	1	*a*–(ii)	49·6	37·7	50·0	56·3
	2	*b*–(ii)	21·5	20·0	16·9	60·8
	4	*c*–(ii)	−9·7	−4·4	−3·8	2·5
	4	*d*–(ii)	−1·2	5·7	3·8	10·2
III	1	*a*–(iii)	30·7	24·6	29·5	52·6
	2	*b*–(iii)	2·0	0·3	3·2	26·8
	5	*c*–(iii)	0·9	−0·8	1·6	13·3
	5	*d*–(iii)	−0·3	−2·4	0·5	1·0
IV	6	*a*–(ii)	44·3	80·5	47·1	52·9
	6	*b*–(ii)	26·2	40·5	50·6	46·7
	6	*c*–(ii)	−3·2	−12·9	−8·0	−13·6
	6	*d*–(ii)	0·1	−2·3	25·2	29·5

In the final outcome, the total number of assays and recorded observations was large (approx. 1,000 cultures in 6 separate experiments) and, accordingly, the full data will not be presented. Instead the growth-promoting activity of the various extracts will be compared by the following procedure:

(i) For each treatment the increase in growth over explants in basal medium of the explants in the medium containing the extract in question was recorded.
(ii) The increment in growth due to the addition of coconut milk to the basal medium was recorded for the same population of explants.
(iii) The extract was compared with coconut milk by expressing the growth increment due to the extract as a percentage of that due to coconut milk (i÷ii×100).
(iv) The same procedure was carried out for cultures grown in the presence of casein hydrolysate.
(v) The whole procedure was duplicated with explants from two carrot roots. The data are presented in Table VIII. The various treatments are indicated by code.

The first noteworthy fact is that there is a highly positive effect of the alcoholic extracts of the tumours on the growth of the carrot explant (series II, *a*–2 and *b*–2). This effect is most marked in the case of the extracts of the 'Young tumours', which were approximately 50 per cent. as active as whole coconut milk in stimulating the carrot explants to grow, and this effect could be detected even though casein hydrolysate was absent. (It should be noted that the *absolute* amount of growth was greater with the extract plus casein hydrolysate, though the relation to the effect of coconut milk was not greatly affected.) The other alcohol extracts that showed positive activity were the extracts from the 'Old tumours', though their ability to replace whole coconut milk was notably less.

The idiosyncrasies of particular carrots, revealed by the effects of casein hydrolysate, are shown by the responses of explants from carrot 'B'. In the case of all the extracts of 'Old tumours' which were tested by the use of explants from this carrot (see *b*–1, *b*–2, *b*–3), the added extracts were very much more effective in presence of casein hydrolysate, showing that the casein hydrolysate reacted synergistically with the extracts, particularly the tumour extract. Even so, however, the main conclusion is clear. The growth-promoting effect of the stem and leaf tissue extracts towards the carrot tissue (B) was either absent or small, the unsupplemented alcohol extract of the tumours (in this case 'Old tumours') alone showed growth-promoting activity (series II, *b*–2 (B)), and this was very strongly enhanced by casein hydrolysate. The alcohol extracts of stem and leaf tissue were either somewhat inhibitory, reducing the growth below their corresponding control, or the degree of positive activity was small compared to that of the 'Young tumours'.

The ability of the water extract of 'Young tumours' to replace coconut milk and produce growth in carrot explants is real, though less than that of the alcohol extract. The water extract of 'Old tumours' was only effective when supplemented by casein hydrolysate, and even then the positive response was obtained only with explants from one carrot (B).

The order in which the extracts of *Bryophyllum* promote growth in the carrot explants was, therefore, Young tumours > Old tumours > Stem, Leaf. The different kinds of tumour extracts were effective in the following order: alcohol extract > water extract > ether extract. In the presence of casein hydrolysate, tumour extract may replace the effect of whole coconut milk to the extent of 50 per cent.

In series IV all the alcohol extracts were again tested simultaneously in one experiment on explants from two carrots. Again these showed some individual idiosyncrasies. Despite this, however, the superiority of the alcohol extracts of the tumours over the stem and leaf tissue in promoting growth in the carrot explants was clearly marked. The effectiveness of the tumour extracts to replace coconut milk was again evident, even though some positive activity appeared with the leaf extracts, provided casein hydrolysate was also added. In this series of assays, in the presence of casein hydrolysate the superiority of extracts made from the younger tumours in their ability to replace coconut milk was not as marked as in series II and it is clear that it is an idiosyncrasy of the carrot which determines whether the effect of casein hydrolysate is additive to, or synergistically accentuates, that growth-promoting effect of the extract which resembles the effect of coconut milk.

These extracts, therefore, show that alcoholic extracts of relatively young tumours of *Bryophyllum* consistently show ability to replace coconut milk to the extent of about 50 per cent. and this occurs whether or not casein hydrolysate is present in the medium. Though somewhat affected by the idiosyncrasies of particular carrots from which the explants were derived, and whether the comparison was made in the presence or absence of casein hydrolysate, the extracts from the oldest tumours were usually less able to replace coconut milk in the carrot assay, though they were invariably more active in promoting growth than similar extracts from stem or leaf under the same conditions. (It should be noted that the old tumours here referred to were much older, larger, and woodier than the oldest referred to in Text-fig. 1.)

The tumour extracts which had positive growth-promoting activity towards carrot explants were negative in the *p*-dimethylaminobenzaldehyde test, although this reagent will readily detect indoleacetic acid at low concentration. Furthermore, in our experience, the primary growth response of the carrot explants as used in this work is not a response to indoleacetic acid. Therefore there is no reason to believe that the growth-promoting substance from the tumours is indoleacetic acid, even though indoleacetic may play a role in the over-all response which leads to tumour formation (Klein and Link, 1952), by acting in combination with a tumour-inducing principle formed by the crown-gall bacteria (Klein, 1953). In a similar manner, 2,4-D and coconut milk growth factors are *both* necessary to stimulate potato tuber tissue to grow as a tissue culture (Steward and Caplin, 1951).

CONCLUSIONS

These data all agree with a general idea that has already been suggested (Steward and Caplin, 1952*b*). As differentiation occurs, the activity in random cell division of the young embryo is brought under control. It is convenient to regard this as due to the presence of an inhibitor of the growth-promoting substances that can be tested by the carrot assay. It is possible that the substances, or mechanisms, that inhibit the effect of the 'coconut milk factor', or its equivalent, during development, may also act as a positive agent that promotes differentiation. Be that as it may, the return of mature cells to the randomly proliferating state may result when the normal balance of growth promoter and inhibitor is disturbed and the growth-promoting, cell-division factor is found to be in excess.

It is therefore of great interest that the extracts of tumours (water extracts and alcohol extracts) contain substances that have the same effect on carrot tissue as the nutritive fluids of immature embryos. Moreover, the tumour extract contains these substances in definitely greater amount than the mature tissues of stem and leaf. The presence of the tumour suffices to cause these growth-promoting substances to persist. Thus plants like *Kalanchöe* and *Bryophyllum* which form tumours with ease may already have the balance of growth-promoting substance and inhibitor adjusted in such a way that it may be very readily disturbed, so as to allow the growth substance to react uninhibited.

The nature of the inhibitory substance or mechanism that in normal, mature, or differentiated tissue inhibits the factor or factors which, like those of coconut milk, impel the cells toward random proliferation, now becomes of great interest because it represents the means of regulatory control over cell proliferation, without which differentiation could hardly occur. Once this regulatory control is dislocated or inactivated, as by the effect of the crown-gall organism, a tumour results. The tumour cells show a condition of imbalance between growth promoter and inhibitor which can be detected by using alcohol or water extracts of these tumours, in place of coconut milk, in the carrot tissue culture assay method.

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EXPLANATION OF PLATE I

Illustrating F. C. Steward, S. M. Caplin, and E. M. Shantz's article on 'Investigations on the Growth and Metabolism of Plant Cells. V. Tumorous Growth in Relation to Growth Factors of the Type found in Coconut Milk.'

Growth and Structure of Carrot Tissue Culture in Different Media[1]

Sections cut 10μ thick; stained safranin-fast green.

(i) to (iv) Photographs, at magnification of 5·5 diameters, of median longitudinal sections through the explant. Long axis of the cylinder, parallel to the axis of the carrot, figures mounted with this direction horizontal.

- (i) Original explant.
- (ii) Explant after 21 days in basal medium.
- (iii) Explant after 21 days in basal medium plus casein hydrolysate.
- (iv) Explant after 21 days in basal medium plus casein hydrolysate plus coconut milk (note air spaces in interior of the culture).

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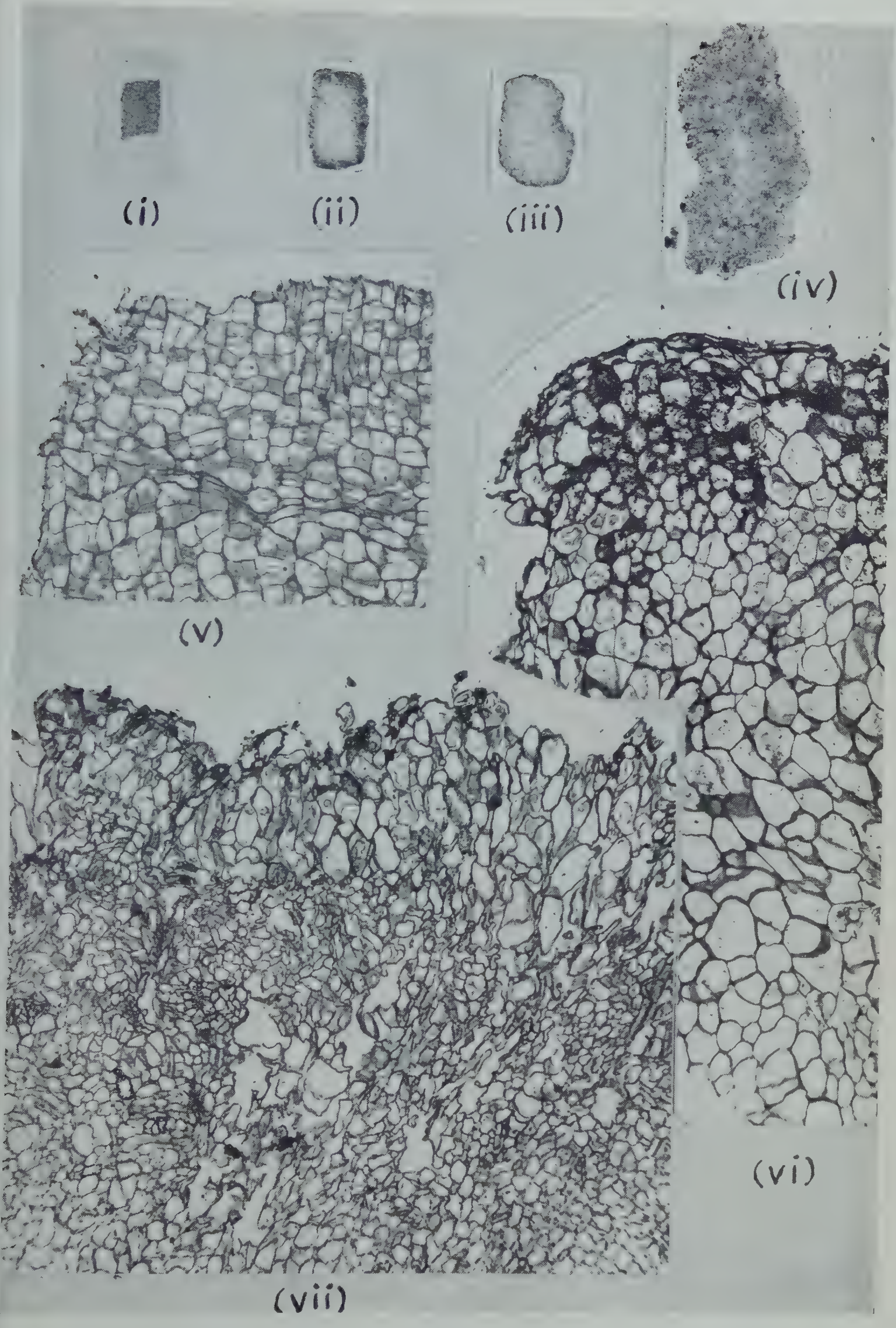
[1] These preparations were made for the authors by Miss J. Sandford under the direction of one of us (F. C. S.).



F. C. STEWARD, S. M. CAPLIN, *and* E. M. SHANTZ

(v) to (vii) Photographs at a magnification of 70 diameters.

(v) Representative portion of surface shown at (i). Note many cells in which divisions had occurred in the carrot root, but the daughter cells had not fully expanded.

(vi) Representative portion of surface shown at (ii). Note that the average cell size is larger than in (v). In the central portion the original cells have enlarged in a disorderly manner, but there is little evidence that cell divisions have occurred throughout the tissue mass. This growth in basal medium, which is a variable function of the individual carrot root, depends mainly on preformed cells which can expand.

(vii) Representative portion of surface shown at (iv). Note the abundance of small cells the air spaces, and some larger cells towards the periphery of the culture.

Investigations on the Growth and Metabolism of Plant Cells

VI. Growth of Potato Tuber Tissue in Culture: The Synergistic Action of Coconut Milk and Some Synthetic Growth-regulating Compounds

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ABSTRACT

A number of substances have been found which can replace 2:4-dichlorophenoxyacetic acid (2:4-D) as a synergist with coconut milk in stimulating the growth of potato tuber tissue in culture. Two of these compounds, viz. α-(2-naphthoxy)- and α-(2:4:5-trichlorophenoxy)-propionic acids, are optically active and their (+)- and (−)-enantiomorphs have also been examined for synergistic activity. In each case the (+)-form was found to be highly active and the (−)-form inactive. Evidence is also presented showing that the inactive (−)-isomer of α-(2-naphthoxy)propionic acid can depress the synergistic activity of the (+)-isomer and it is suggested that this occurs by competitive antagonism.

INTRODUCTION

IN investigations at Wye on synthetic plant growth-regulating substances a large number of aryl- and aryloxy-alkylcarboxylic acids have been prepared and their activity assessed by various methods for studies on the relationship between molecular structure and biological activity. The results have recently been summarized (Wain, 1954).

In the investigation of the growth of explants of mature parenchymatous tissue, work started at the University of Rochester and continued at Cornell University disclosed growth-promoting qualities in coconut milk for this type of material. This was particularly true of carrot tissue, which is convenient to use in an assay system under the recommended conditions (Steward et al., 1952; Caplin and Steward, 1952). While the cells of carrot and Jerusalem artichoke tuber were found to grow rapidly in a basal medium supplemented by coconut milk alone (Steward and Caplin, 1952), potato tuber tissue responded to coconut milk only in the presence of an appropriate concentration of 2:4-D (2:4-dichlorophenoxyacetic acid) (Steward and Caplin, 1951).

The synergistic action of 2:4-D and coconut milk, disclosed by their effects on potato tuber tissue, suggested that this would furnish another and quite different system on which to test the activity of certain synthetic growth regulators. It also became of interest to determine whether substances possessing an asymmetric carbon atom might show a similar high synergistic activity when

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tested with coconut milk. That the stereoisomers of such optically active growth substances can induce markedly different growth responses had been demonstrated (Smith and Wain, 1952) and, further, it had been shown at Wye that the growth-regulating activity of one stereoisomer as assessed by the *Avena* cylinder elongation test may be reduced in presence of an excess of its inactive enantiomorph, an effect attributed to competitive antagonism (Smith et al., 1952). It was therefore logical to investigate whether the (+)- and (—)-forms of a growth substance exerted different synergistic effects with coconut milk and whether any competitive antagonism between the stereoisomers could operate.

The chemical work was carried out at Wye College and the tests using the tissue culture technique were performed at Cornell University. The plan of the experiments was to grow explants of potato tuber tissue in liquid medium under the conditions which have been described for carrot root explants. Explants were grown in the basal medium (Caplin and Steward, 1952) supplemented with adequate concentrations of coconut milk and the growth-regulating substances in question. As controls, the behaviour of potato explants from the same tubers, randomly sampled from the same batch, was determined in the basal medium and in the basal medium supplemented by coconut milk and in some cases by 2:4-D and by 2:4-D and coconut milk in combination. At the end of the experiment the mean fresh weight for each treatment of the cultures was determined and the standard deviation estimated from the replicates, necessarily rather small in number on account of the limited amount of some of the compounds that could be used.

MATERIALS AND METHODS

Aryloxyalkylcarboxylic acids. These were prepared by condensing the sodium salt of the appropriate phenol with either ethylbromoacetate or ethyl α-bromopropionate in alcoholic solution and hydrolysing the product. Details of the preparation of α-(2-naphthoxy)propionic acid have already been given (Smith et al., 1952).

1:2:3:4-tetrahydro-1-naphthoic acid. 1-naphthoic acid, prepared from naphthalene via 1-bromonaphthalene and 1-cyanonaphthalene, was converted to its 1:2:3:4-tetrahydro-derivative by reduction with sodium in amyl alcohol by the method of Sowinski (1891). The product (76 per cent. yield) was recrystallized twice from light petroleum; colourless prisms m.p. 84–85° C.

Resolution of optically active aryloxy acids. Details of the resolution of α-(2-naphthoxy)- and α-(2:4:5-trichlorophenoxy)-propionic acids have been published (Smith and Wain, 1951; Smith et al., 1952).

BIOLOGICAL METHODS

Potato explants. These were removed aseptically as uniform cylinders from potato tubers after the manner described previously for carrot roots (Caplin and Steward, 1952). They were grown under standardized conditions in the specially designed culture tubes using the apparatus described previously (Steward et al., 1952). Each tube contained a modified White's nutrient solution (Caplin and Steward, 1952) supplemented in certain cases with coconut milk and growth substance. The pH of all solutions was adjusted to 6·4.

Activity of various growth substances. The following compounds were tested for activity as synergists of coconut milk for inducing growth in potato tuber explants:

1. α-(2-naphthoxy)propionic acid, m.p. 107–108·5° C.
2. α-(2-naphthoxy)phenylacetic acid, m.p. 153° C.
3. α-(2:4:5-trichlorophenoxy)propionic acid, m.p. 179–180·5° C.
4. 1:2:3:4-tetrahydro-1-naphthoic acid, m.p. 84–85° C.

Also tested in the same experiment were the following more commonly used growth substances:

5. 2:4-dichlorophenoxyacetic acid, m.p. 141° C.
6. 3-indolylacetic acid, m.p. 164–165° C.
7. 2-naphthoxyacetic acid, m.p. 155–156° C.
8. 3-indolylbutyric acid, m.p. 124° C.

TABLE I

Growth in mg. of 2·6 mg. Potato Tuber Explants in a Medium containing Coconut Milk and Added Synergists. Growth Period 34 days

	Concentration.			
Treatment.	30 p.p.m.	6 p.p.m.	1 p.p.m.	0·1 p.p.m.
Compound 1	1·9±0·1	11·9±2·3	12·7±2·7	4·5±1·3
,, 2	1·9±0·1	1·8±0·1	4·4±0·2	3·9±0·1
,, 3	1·8±0·1	14·9±3·9	9·2±4·7	4·2±0·9
,, 4	24·4±7·2	14·3±0·4	8·9±1·6	3·2±0·2
,, 5	5·8±3·6	17·9±6·3	12·9±1·7	6·6±1·0
,, 6	1·9±0·1	1·8±0·1	4·6±2·0	3·0±1·1
,, 7	2·5±1·3	12·0±7·2	13·4±4·7	7·5±2·2
,, 8	2·0±0·2	16·2±1·4	8·9±5·1	2·7±0·2

Controls

(*a*) Basal solution only 2·9±0·5.
(*b*) Basal+5% CM 2·9±0·1.

2:4-D was included as a general standard of reference being a highly active synergist with coconut milk. 3-indolylacetic acid was used to indicate the activity that might be expected from the naturally occurring auxin.

Compounds 1–4 were tested at 600, 60, 30, 6, 1, and 0·1 p.p.m. while compounds 5–8 were tested at 30, 6, 1, and 0·1 p.p.m. only. All treatments except the controls on basal nutrient medium contained 5 per cent. whole coconut milk. The initial weight of the explants was 2·6 mg. The average final fresh weight of 5 explants for each treatment after 5 weeks of growth is given in Table I; since compounds 1–4 were definitely toxic at 600 and 60 p.p.m. results for the four lower concentrations only are recorded.

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The results showed that all of these substances except α-(2-naphthoxy)phenylacetic acid and 3-indolylacetic acid (compounds 2 and 6) can replace 2:4-D (compound 5) in its synergistic action with coconut milk upon the growth of the potato tuber. All other compounds at 6 p.p.m. exerted an activity comparable to that of 2:4-D though the activity of the tetrahydronaphthoic acid (compound 4) persisted to a higher concentration level. The apparent inactivity of the natural auxin, 3-indolylacetic acid, is of interest. It should be noted that in some other experiments carried out at the Cornell Laboratory, not recorded here, 3-indolylacetic acid has produced a positive response though always significantly less than those produced by a variety of other synthetic auxin-like compounds. However, as shown below, the data of Table I only serve to *detect* activity. They may not properly reflect the relative activity of those substances that contain an asymmetric carbon atom, for, following the work at Wye, it became evident that the (+)- and (−)-forms may have differential activity (Smith and Wain, 1951) and even antagonistic action (Smith et al., 1952).

EXPERIMENTS WITH STEREOISOMERS

The (+)- and (−)-forms of α-(2-naphthoxy)propionic acid (compound 1) and of α-(2:4:5-trichlorophenoxy)propionic acid (compound 3) were examined separately for synergistic activity, 1:2:3:4-tetrahydro-1-naphthoic acid (compound 4) being included in the same test. Four tubes containing 3 explants per tube were used for each treatment. The results, recorded in Table II, represent the average final fresh weight after a 5-week growth period of 9–12 explants for each treatment. All of the treatments except two controls (*a* and *c*) contained 5 per cent. whole coconut milk.

TABLE II

Contrasted Effect of Enantiomorphs. Growth in mg. of 2·6 mg. Potato Tuber Explants in a Medium containing Coconut Milk and Added Synergists. Growth Period 32 days

	Concentration.			
Treatment.	30 p.p.m.	6 p.p.m.	1 p.p.m.	0·1 p.p.m.
Compound 1 (+)-form	6·2±2·3	38·8± 9·6	16·2±2·8	8·9±0·6
„ 1 (−)-form	3·3±1·1	4·0± 0·4	8·1±7·2	3·7±0·7
„ 3 (+)-form	3·7±0·3	18·9±13·4	25·5±8·5	11·2±1·2
„ 3 (−)-form	3·7±0·2	5·0± 0·7	7·2±1·4	6·3±0·5
„ 4	7·2±2·3	24·4± 2·8	8·9±0·5	6·5±1·5

Controls

(*a*) Basal medium only	3·2±0·9.
(*b*) Basal+5% CM	6·6±1·7.
(*c*) Basal+2:4-D 6 p.p.m.	9·3±5·9.
(*d*) Basal+CM+2:4-D	23·6±8·1.

The results show clearly that the (+)-forms of compounds 1 and 3 are active while their respective (−)-forms are not. When resolved, compound 1 is more active than both compound 4 and 2:4-D (control d).

COMPETITIVE ANTAGONISM

The fact that the growth-regulating activity of one stereoisomer, as assessed by the *Avena* cylinder elongation test, may be reduced in presence of an excess of its inactive enantiomorph has already been demonstrated (Smith et al, 1952). Accordingly the possibility of such antagonism between the enantiomorphs of compound 1 was investigated in the present work by keeping the concentration of the (+)-form constant at a level of 6 p.p.m. which is favourable for growth, and adding varying amounts of the (−)-isomer. Tissues were grown with 5 per cent. whole coconut milk for 5 weeks, using 4 tubes per treatment and 3 explants per tube, as in the second experiment. The Results are given in Table III.

TABLE III

Effect of Increasing Concentrations of (−)-α-(2-naphthoxy)propionic Acid on the activity of the (+)-isomer. Growth in mg. of 2·6 mg. Potato Tuber Explants in a Medium containing Coconut Milk. Growth Period 35 days

Treatment No.	Concn. of compd. 1		(−)/(+) ratio.	Average final fresh weight.
	(+)-form.	(−)-form.		
1	6·0 p.p.m.	0·0 p.p.m.	0·0	27·1± 6·0
2	6·0 p.p.m.	0·96 p.p.m.	0·16	31·7±10·1
3	6·0 p.p.m.	2·4 p.p.m.	0·4	26·9±10·2
4	6·0 p.p.m.	6·0 p.p.m.	1·0	9·7± 9·1
5	6·0 p.p.m.	15·0 p.p.m.	2·5	8·7± 5·3
6	6·0 p.p.m.	37·5 p.p.m.	6·25	2·6± 0·1

Controls

(*a*) Basal solution only	2·9±0·2.
(*b*) Basal+5% CM	4·4±1·2.
(*c*) Basal+2:4-D 6 p.p.m.	7·4±4·0.
(*d*) Basal+CM+2:4-D	27·2±8·4.

In interpreting this experiment one needs to be assured that growth would occur at concentrations of the (+)-form of compound 1 higher than 6 p.p.m., because the depressed growth at higher concentrations of the inactive (−)-form might be due to some toxicity unrelated to the more specific physiological action of its enantiomorph. The *absence* of such toxic action, however, is difficult to prove. Hence antagonism between enantiomorphs can be assumed to occur only in those cases where the growth response to a mixture of enantiomorphs is both (1) less than the response to a concentration of the active form alone equal to the total concentration of the mixture, and (2) less than the response to a concentration of the active form alone equal to its individual concentration in the mixture.

So far as it goes, some evidence is furnished by treatment No. 3 (Table III) at a total concentration of 8·4 p.p.m., and it will be noted that the effect of the higher total concentration on growth, though downward, is not significant. Table II shows that at a concentration of 30 p.p.m. the (+)-form of compound

1 is inactive, so treatments 5 and 6 might be a response to total concentration of compound 1, without regard to its rotation. It is, however, highly unlikely that the significantly depressed growth at a total concentration of 12·0 p.p.m. in treatment 4, using the (+)- and (−)-forms in equal concentration, is due to anything other than competitive antagonism of the two forms in their effect upon the growth of potato cells. The *progressive* reduction in the mean fresh weight as the concentration of the (−)-form increased from the low value of 0·96 p.p.m. in treatment 2 to 37·5 p.p.m. in treatment 6 is consistent with the idea that the inactive (−)-form has an inhibitory action upon the active (+)-form.

Although the above results show that the substituted propionic acids are active in this growth test only in the *dextro*-rotatory form, the evidence of competitive inhibition by the *laevo*-rotatory form is open to the criticism that the observed effects might be due to total concentration of the enantiomorphs and not to competition between them. Further investigations were carried out to eliminate this possible criticism.

TABLE IV

Tests for Competitive Inhibition between Enantiomorphs of α-(2-naphthoxy)-propionic Acid, Second Experiment. Growth in mg. of 3 mg. Potato Tuber Explants in a Medium containing Coconut Milk. Growth Period 25 days

Tuber 'A'.		Concn. of (+)-form in p.p.m.				
		0.	4.	8.	12.	16.
Concn. of (−)-form in p.p.m.	0	11·0±3·2	72·3±10·9	68·8±11·0	60·1±13·2	64·9±5·2
	4	5·1±1·1	64·5±10·5	66·3± 8·8	70·6± 2·1	—
	8	6·6±3·1	52·6±10·9	67·1± 6·5	—	—
	12	5·9±1·6	47·0± 6·1	—	—	—
	16	4·5±1·4	—	—	—	—

Control (6 p.p.m. 2:4-D) 56·7±7·5

Tuber 'B'.		Concn. of (+)-form in p.p.m.				
		0.	4.	8.	12.	16.
Concn. of (−)-form in p.p.m.	0	5·5±3·3	63·8±14·2	70·9± 4·8	46·3±12·2	56·0±13·4
	4	7·4±4·9	47·5± 8·7	59·3±13·2	55·7±14·4	—
	8	4·6±1·9	43·5± 5·3	49·7± 8·1	—	—
	12	4·3±1·4	40·9± 2·1	—	—	—
	16	3·8±0·3	—	—	—	—

Control (6 p.p.m. 2:4-D) 53·5±8·9

A second experiment was therefore carried out using the enantiomorphs of α-(2-naphthoxy)propionic acid. The medium consisted of the basal nutrient solution plus 10 per cent. coconut milk to which was added various concentrations of the two enantiomorphs, separately and in combination, in such a way (as shown in Table IV) that one could distinguish between the effects due to total concentration and effects due to the operation of the enantiomorphs singly. For example, in the table, the response of the potato tissue in basal medium plus coconut milk, supplemented by 0, 4, 8, 12, and 16 p.p.m. of each form, is shown. The response of the tissue to all possible combinations up to a maximum total concentration of 16 p.p.m. is also recorded. Thus the growth response to any mixture can be compared with responses to each of the two forms, taken separately, at the concentration at which either occurs in the mixture. Comparisons can also be made between the effects of enantiomorph mixtures and the effect of either form separately at the *total* concentrations of the mixture.

In the experiment planned as shown above, 3 mg. explants removed from two potato tubers of the same stock were grown for 25 days. In each treatment the recorded observations consist of the mean growth (mg. fresh weight) obtained from 5 replicate explants together with the standard deviation of this mean. The data obtained on explants from two potato tubers are recorded to show their essential similarity.

These data again show clearly the inactivity of the (—)-form and the activity of the (+)-form, which, in combination with coconut milk, promotes the growth of the potato tissue. At a given concentration of the (+)-form, e.g. at 4 p.p.m., successive increments of the (—)-form tend to decrease the growth response of the potato tissue. At a total concentration of 16 p.p.m., made up of 12 p.p.m. of the (—)-form and 4 p.p.m. of the (+)-form, the total growth was definitely less than at either 4 or 16 p.p.m. of the (+)-form alone. At higher concentrations of the (+)-form, e.g. 8 and 12 p.p.m., the influence of the (—)-form was inevitably less marked and any apparent competitive effect was not significant.

From these experiments it seemed that the most conclusive evidence of competition between the two forms would be obtained at a total concentration which would not limit the growth of the tissue and yet would permit ratios of the (—)-form to the (+)-form to be as large as possible. Following the same general plan, a further experiment was carried out with explants from two potato tubers, using a maximum total concentration of 6 p.p.m. with individual concentrations of 1, 2, 3, 4, 5, and 6 p.p.m. of the two enantiomorphs. This permitted the ratio of the (—)- to the (+)-form to reach a maximum value of 5.

In general, this experiment did not show any significant evidence of competitive inhibition by the (—)-form, even at the highest ratio of 5 to 1 and detailed results are not presented. The reason for absence of competitive inhibition may be that the available sites are adequate for both forms at these lower total concentrations, so that no real competition occurs. However, this experiment did show that the (+)-form was quite effective at 1 p.p.m. in promoting growth of the potato tissue. Since the earlier experiment had shown that the potato tissue also tolerated the (+)-form at 16 p.p.m., a final experiment was carried out using a total concentration of 16 p.p.m. with ratios as great as 15 to 1 of the (—)- to the (+)-form. These data are found in Table V.

TABLE V

Tests for Competitive Inhibition between Enantiomorphs of α-(2-naphthoxy)-propionic Acid, Third Experiment. Growth in mg. of 3 mg. Potato Tuber Explants in a Medium containing Coconut Milk. Growth Period 25 days

Concn. in p.p.m.		Ratio	Growth response.	
(+)-form.	(−)-form.	(−)/(+).	Tuber 'A'.	Tuber 'B'.
0	0	—	12·4 ± 2·0	12·6 ± 1·7
1	0	—	40·6 ± 8·3	47·0 ± 4·8
2	0	—	42·6 ± 10·1	52·9 ± 13·5
4	0	—	51·2 ± 9·6	52·9 ± 20·5
16	0	—	35·5 ± 4·7	41·6 ± 8·0
1	15	15	29·5 ± 4·8	32·1 ± 4·6
2	14	7	32·1 ± 2·8	33·0 ± 3·3
4	12	3	30·9 ± 5·4	38·9 ± 4·3

From Table V it can be seen that good growth responses were obtained at all concentrations of the (+)-form from 1 to 16 p.p.m., although the highest concentration appears to be slightly inhibitory. It is also apparent that in all treatments containing a mixture of enantiomorphs growth was depressed below these control values. In many cases the reduction of growth is of slight statistical significance. However, since the regular pattern occurs with explants from two different tubers and a similar response has been observed in three different experiments, the cumulative evidence for competitive antagonism between the active and inactive enantiomorphs is strong, though not so conclusive as that obtained by Smith et al (1952) in the *Avena* cylinder elongation test where higher mole ratios were employed.

In summary, the role of 2:4-dichlorophenoxyacetic acid as a synergist with coconut milk which stimulates the growth of potato tuber tissue in culture (Steward and Caplin, 1951) is not specific. It is replaceable by a variety of other synthetic substances whose growth-regulating effects in other systems have been investigated by Wain and his collaborators. Two of these, α-(2-naphthoxy)propionic acid and α-(2:4:5-trichlorophenoxy)-propionic acid, were more effective than 2:4-dichlorophenoxyacetic acid. It is evident also that these substances are active only in the *dextro*-rotatory form. Their *laevo*-rotatory enantiomorphs are not only inactive, but may show competitive antagonism for the *dextro*-rotatory forms similar to that which has been observed at Wye College using other biological tests.

Although the growth of potato tuber tissue stimulated by the synergistic action of coconut milk and a substance like 2:4-D as described in these experiments was considerable, it still did not attain the rate of growth shown by carrot explants in the same basal medium supplemented by whole coconut milk alone. It is now known that the growth-promoting qualities of the whole coconut milk are complex (Shantz and Steward, 1952). They are due to a non-specific part replaceable by casein hydrolysate, and to a part, specific for coconut milk, which consists of several different, but separately active, substances, three of which have been isolated. Therefore the growth of potato tuber tissue which requires both coconut milk and a substance like 2:4-D may be still more responsive if other growth-promoting substances are supplied. Such combinations may be required to release the full ability of the potato cells to grow. Such additional substances may compensate for deficiencies in the coconut milk where the growth of potato tissue is concerned, or they may affect centres, other than those affected by coconut milk and 2:4-D, which limit growth of the cells. Between the growth rate of potato tissue already achieved and the growth rate of tissue from the carrot root or the artichoke tuber there seems to be room for still other responses of this kind. Some unpublished evidence available in the Cornell Laboratory (F. C. S. and E. M. S.) indicates that this is, in fact, the case.

Full explanations of these effects require further study. An attractive hypothesis arises from the work of Steward and Caplin. These authors found that potato tuber tissue contains an inhibitory substance or mechanism for the coconut growth factor(s). Thus, the substances like 2, 4-D may exert their synergistic effect by acting as antimetabolites for the naturally occurring inhibitor, thus allowing the otherwise mature cells to respond to the coconut milk factor when this is supplied.

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The Estimation of Ground Cover by the Point Quadrat Method

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ABSTRACT

In studying pasture composition it has been found that with certain species the numbers of individual plants per sample quadrat followed the negative binomial distribution. Others, however, where the isolation of individuals is difficult, such as grasses showing a creeping type of growth, did not appear to follow this distribution. Another method of pasture analysis is therefore required, and the paper describes an examination of a pasture in Kenya by the Point Method described by Levy and Madden. It is suggested that for pastures less uniform than those normally found in Great Britain there should be a greater distance between the points of the apparatus for it to be efficient.

INTRODUCTION

THE importance of reliable information on the composition of pasture and on its changes under various treatments has been stressed by many writers. There are three basic techniques by which pasture composition can be measured, namely, productivity by weight of the different species in the harvested herbage, plant counts, and ground cover. For studying long-term changes in pasture composition the first of these measurements will be dependent on the immediately preceding climatic conditions which may mask any long-term effects of treatments. Plant counts and ground cover will probably give a better indication of the more lasting effects of the different treatments. The former has been discussed in an earlier paper (Robinson, 1954) using data collected by Steiga (1930). For most species it was found that the numbers of plants counted in quadrats of 1 metre square were distributed according to the negative binomial. Reference, however, should be made to a short paper by David and Moore (1954) in which they suggest that the distributions obtained do not necessarily follow the negative binomial. Their arguments are possibly suggestive that other distributions may be better in certain cases, but are not conclusive. The actual distribution of some species examined did differ significantly from the theoretical expectations calculated on the basis of the negative binomial, but these differences existed only where the separation of plant communities into individuals was difficult, and was mainly confined to grasses showing a creeping type of growth. With such species the use of plant numbers to determine pasture composition may give misleading results, and it would therefore be better to use the last of the three basic techniques, i.e. ground cover.

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It was shown in the earlier paper that the ground cover of three species which were examined followed the Pearson Type I distribution: a continuous distribution varying from zero, indicating complete absence of the species, to unity, indicating complete cover. Steiga had determined the ground cover by a detailed mapping of the quadrats, which would, of course, lead to a continuous distribution. The data for the present paper, however, were collected by the point quadrat methods, first described by Levy and Madden (1933). Using this method the ground cover will follow the binomial distribution, a non-continuous type.

The apparatus used by Levy and Madden consisted of a metal framework carrying a row of ten steel pins at intervals of 2 in. The frame was placed at random in the sample area and the numbers and types of species hit by the pins were recorded. With a sufficient number of samples, the percentage ground cover can be determined. Two types of apparatus, both being slight modifications of this, were employed in the present investigation to test their relative efficiency. The first was simply a stout length of wood through which ten 6-in. nails had been driven 2 in. apart. The second was basically the same as the original, except that the pins were 4 in. apart instead of 2 in. These two types of apparatus will be denoted by 'nails' and 'pins' respectively throughout the paper.

PRACTICAL DETAILS

The area under investigation was an experimental plot of approximately 1 acre on the Kitale Grassland Experiment Station. Kitale is in a large maize-growing area of Kenya at about 6,000 ft. above sea-level, with an average rainfall of some 40 in. per annum. The plot had not previously been grazed and the grass was very tall. The samples were taken at the beginning of March, before the long rains, and the vegetation consisted mainly of dead stem and leaf, which was to be burnt for experimental purposes.

From the practical point of view the nails were easier to carry about in the field, but with the vegetation at this stage the shorter projections of the nails made it rather difficult to clear away the well-knit cover when recording the number of hits. In doing this it was also very difficult to prevent the board from moving slightly, so that the sample obtained was not the strictly random one that it should have been. In a pasture of young growth these practical difficulties are unlikely to arise.

The framework for the pins was, of course, somewhat larger and more cumbersome for carrying over the plot. Its size, however, was also an advantage, for when placed on the ground in the sampling position it did not move when the long dead grass was cleared and two hands were left free for the operation. The apparatus used in this investigation suffered from two minor defects: the pins showed some slight tendency to bend, and the ends were not pointed, so that sometimes when the pins dropped they would slide off the grass roots on to bare earth. This, however, did not happen very often, and where there was some doubt the pins were drawn up and allowed to fall again, and the point of first contact, not the resting position, was recorded.

It was unfortunate that the pasture was not at a younger stage of growth. As it was, in the clearing process the grass would sometimes break off from the root which could then give rise to some doubt in the identification of the species with only the root remaining. It was also unfortunate that it was impossible for one person to complete the identification of all species during the sampling. With the difficult type of vegetation, and the resulting errors in identification, it is to be expected that there will be differences between investigators in the distribution of certain species. Such differences between investigators should not, however, occur when the total ground cover of all species is considered, and this has been used for the critical test of the merits of the two types of apparatus. In addition, one species, *Loudetia*, has been used for further illustration, as it is one of the easier plants to identify.

METHOD OF SAMPLING

The plot was roughly rectangular in shape, and fencing-poles had just been erected around the perimeter. One investigator, A, took two transects parallel to the long sides of the rectangle for obtaining the samples, using the pins. At every pace the apparatus was placed immediately in front of the investigator and at right angles to the path of the transect. The number of hits and the species involved were then noted. This was done 140 times, thus obtaining 1,400 points. The transects used for nails were at right angles to the long sides of the rectangle, and a further investigator, B, identified the species. In this case 600 points were obtained.

It was then thought that one pace between samples was not sufficient, for in obtaining samples covering the whole area a very large number would be involved. The number of paces between samples was therefore increased to 6. Investigator C was then asked to take over the sampling. The transects chosen were demarcated by the fencing-poles along the long sides of the rectangle. A zigzag pattern covering the whole area was obtained by using alternate posts on each side for pins and a similar pattern using the remaining posts was obtained for nails. In this way 1,090 points were obtained for pins and 1,470 for nails.

GROUND COVER

The results obtained by the three investigators are given in Table I. Investigator A took two transects with pins, and the results are given separately in the table. The data were transformed into degrees for statistical analysis, and the difference between the means of the two transects was tested. The transects of investigator C were grouped together in two classes by taking alternate transects of the zigzag pattern, and the data were again transformed into degrees for analysis. The means and the variances on the transformed scale are given in Table II. In all three cases (i.e. the two transects of A using pins and the two groups of transects of C using pins and nails) there was no difference between the two means. There was also no difference between the means obtained by the three investigators, and there was very good agreement between the two methods of obtaining samples.

TABLE I

Ground Cover

	Nails.					Pins.					
Investigator.	B.	C.		Total.	Expected.	A.		C.		Total.	Expected.
Number of hits.		I.	II.			I.	II.	I.	II.		
0	5	12	5	22	19·7	3	4	7	4	18	21·5
1	18	26	15	59	52·2	14	18	13	13	58	54·9
2	20	24	14	58	62·3	11	23	15	11	60	63·1
3	11	12	11	34	44·1	8	10	17	17	52	42·9
4	2	8	9	19	20·5	3	5	3	3	14	19·2
5	3	3	4	10	6·5	1	—	2	4	7	5·9
6	0	3	1	4	1·4	—	—	—	—	—	1·5
7	1	—	—	1	0·3	—	—	—	—	—	
Over 7	—	—	—	—		—	—	—	—	—	
Total	60	88	59	207	207	40	60	57	52	209	209
Mean No. of hits	2·02	1·99	2·34	2·10	2·10	1·92	1·90	2·03	2·27	2·03	2·03

The main differences to be observed lie in the amount of variation. Investigators A and B, in taking samples only one pace apart, covered a much smaller area than that covered by C. This smaller area should then be more homogeneous in character than the whole plot, which should result in the variance being smaller. Table II shows that this was true for both nails and pins; the differences, however, were not statistically significant. It is nevertheless interesting to note that the differences which did exist were in the direction to be expected.

TABLE II

Ground Cover. The Means and Variances of Samples: Data Transformed into Degrees

	Nails.				Pins.				
	B.	C.		All samples.	A.		C.		All samples.
		I.	II.		I.	II.	I.	II.	
Mean . .	24·98	24·05	27·07	25·18	24·46	24·51	24·74	26·80	25·14
Variance . .	123·97	163·58	144·50	146·86	103·70	84·91	134·35	120·64	110·80

When binomial data are transformed into degrees the approximate variance on the new scale is $821/n$, where n is the number of objects involved at each sampling (e.g. see Bartlett, 1947). In this case there are 10 points recorded at each sampling, so that $n = 10$, and the approximate variance of the new scale would be 82·1. All variances, except for transect II of investigator A using pins, are considerably higher than this, which should indicate a large degree of heterogeneity. This approximation to a constant variance, however, is misleading when n, the number of points used, is small, and p, the probability of obtaining a hit on a plant, is not near to 0·5. This is clearly shown in Table III, which gives the actual values for the variance on the transformed scale for different values of n and p. The table of variances is symmetrical about the value $p = 0·5$; in other words, the values for $p = 0·6$ are the same as those for $p = 0·4$. The second half of Table III gives the means obtained on the transformed scale. It should be noted that these are not necessarily equal to the transform of the mean on the original scale.

TABLE III

The Variance and the Mean, on the Transformed Scale, of the Binomial Distribution for Various Values of p, the Probability of obtaining a Hit on a Plant, and n, the Number of Points used

n. *p*.	0·5.	0·4.	0·3.	0·2.	0·1.	Approximate variance.
			VARIANCE			
5	253·4	261·4	279·0	282·8	222·3	164·2
10	94·0	96·6	106·4	125·9	136·0	82·1
20	43·4	43·5	44·5	48·8	63·5	41·0
30	28·2	28·4	28·7	29·9	36·7	27·4
			MEAN			
5	45·0	37·9	30·4	22·0	12·0	
10	45·0	38·8	32·2	24·6	14·7	
20	45·0	39·1	32·8	25·9	16·8	
30	45·0	39·1	33·0	26·2	17·6	

The percentage ground cover is approximately 20 per cent., so, from Table III, the variance on the transformed scale should be about 126. Reference to Table II will show that this figure is generally in closer agreement with the results actually obtained. The variances obtained from the samples taken by investigator A are much lower than this expected value. The reason would probably lie in the close proximity of the samples; with the small area covered, the ground cover would tend to be more homogeneous, with more readings around the mean, resulting in a smaller variance.

Further reference to Table II will show that the variance obtained with nails is higher than that obtained with pins. Bartlett's test for the homogeneity of variances was used, and it was found that the difference was significant at the 5 per cent. probability level. This result was not unexpected, and may be explained by the association existing between points in close proximity.

With nails, the points are only 2 in. apart, so that the ground covered at each sampling is small. With plants having a single stem this would not matter, but with grasses, and also other plants, having a crown covering a fairly large area of ground, the distribution will not be random. There will be a definite association, so that when one hit is obtained there is a likelihood of obtaining more. In other words, with association, the extremities in the distribution will have a greater frequency than values near the mean. This can be quite clearly seen in Table I. With pins the actual numbers obtained are alternately higher and lower than the expected numbers, but with nails the extremes of 0, 1, 5, 6, and 7 classes have actual frequencies greater than expectation, while the middle classes of 2, 3, and 4 have lower actual frequencies. With the greater number of readings at some distance from the mean, the variance will be increased. This means that a greater number of samples will be required if nails are used to obtain the same degree of accuracy.

Pins show very good agreement with the expected binominal distribution. Where such agreement occurs the number of samples required to obtain a given accuracy may be determined from Table III. If it is desired that the variance of the mean should be v per cent. of the mean, then the formula for N, the number of samples required, is

$$N=\left\{\frac{100\sigma}{vm}\right\}^2,$$

where m is the mean on the transformed scale and σ^2 is the variance on the transformed scale. For example, if the ground cover is 20 per cent., then the mean and variance on the transformed scale will be approximately 24·6 and 125·9. With $v = 5$ per cent., then the number of samples required is approximately 83, i.e. 830 points.

The point quadrat method has been compared with the percentage area method by West (1937). An apparatus similar to the nails used in this investigation was used, but instead of placing it in a predetermined position it was thrown, and where it came to rest if was turned over so that the 10 points came into contact with the ground. It is now recognized that throwing hoops and other apparatus of this kind does not produce a random sample. When thrown the nails of this apparatus would catch on the tougher and larger plants, and a sample biased in favour of these plants would be obtained. West found, for example, that the bare bulbous stem bases of *Digitaria tricholaenoides* secured more hits than would be expected from a comparison with the results obtained by the percentage area method. In addition to the bias introduced by this method of sampling, a non-random distribution resulting from the points being only 2 in. apart would also be obtained. It is not surprising, therefore, that the results obtained by West from the two methods should show some disagreement, but it is wrong to conclude that the point quadrat method of sampling gives inaccurate results for the comparison is not a valid one in his case.

LOUDETIA

The frequency of hits on *Loudetia* are given in Table IV. Here the expected difference between investigators is shown very strongly with pins; but with nails somewhat similar results were obtained. The two sets of transects of investigator C using both pins and nails again showed no significant difference, and the two transects of investigator A using pins showed good agreement. The means and variances on the transformed scale are given in Table V. It was further found that investigators A and B obtained comparable results for nails and pins, and investigator C obtained comparable results by the two methods.

TABLE IV

Distribution of Loudetia

	Nails.					Pins.					
Investigator.	B.	C.		Total.	Expected.	A.		C.		Total.	Expected.
Number of hits.		I.	II.			I.	II.	I.	II.		
0	23	43	27	93	75·5	13	15	29	24	81	84·0
1	20	24	12	56	80·1	17	33	19	19	88	80·3
2	12	16	12	40	38·3	9	8	5	6	28	34·2
3	3	4	6	13	10·8	1	4	4	2	11	8·8
4	1	1	1	3	2·1	—	—	—	1	1	1·5
5	1	—	0	1	0·2	—	—	—	—	—	0·2
6	—	—	1	1		—	—	—	—	—	
Over 6	—	—	—	—		—	—	—	—	—	
Total	60	88	59	207	—	40	60	57	52	209	—
Mean No. of hits	1·03	0·82	1·08	0·96	—	0·95	1·02	0·72	0·79	0·87	—

The results so far, apart from the difference between investigators A and C using pins, are the same as those obtained with ground cover. The similarity is carried further in the comparison of the actual distribution and the variances obtained. There was no statistically significant difference between the variances obtained by the two investigators in either nails or pins, but again those obtained by A and B, where the samples were only one pace apart, were on the average slightly lower than those obtained by C. The difference between the two types of apparatus again proved significant at the 5 per cent. level of probability.

TABLE V

Loudetia. Means and Variances on the Transformed Scale

	Nails.			Pins.			
	B.	C.		A.		C.	
		I.	II.	I.	II.	I.	II.
Mean . .	14·5	11·8	14·0	14·6	15·9	10·8	11·8
Variance . .	160·8	150·8	200·5	119·4	103·6	138·1	140·2

The higher variance with nails is again due to the excess of readings at the extreme of the range—in this case mainly in the zero class. This can be clearly seen in Table IV. The distributions obtained with pins by both investigators showed no large disagreement with the theoretical expectations.

From Table III it will be seen that the expected variance, when the mean is approximately 10 per cent., is 136·0, which is very close to the values obtained by C, and somewhat higher than those obtained by A. The number of samples required to obtain a given accuracy can again be calculated using the formula already given for ground cover. With v again being 5 per cent., the number of samples required will be approximately

$$N \doteq \left\{\frac{100}{5\times 14{\cdot}7}\right\}^2 \times 136{\cdot}0$$
$$= 252,$$

where 14·7 is the mean on the transformed scale when the mean ground cover is 10 per cent. Or alternatively, if the number of samples considered necessary to obtain the ground cover with a given accuracy is used, the accuracy of the determination in this case can be measured. From the above formula it will be found that

$$v = \frac{100\sigma}{m\sqrt{N}}$$
$$= \frac{100}{14{\cdot}7}\sqrt{\frac{136{\cdot}0}{83}}$$
$$= 8{\cdot}7 \text{ per cent.}$$

The small increase in accuracy by using 252 samples instead of 83 is probably not sufficient to warrant a large increase in the number of samples taken.

These formulae cannot be used without prior knowledge of the percentage cover. It is therefore necessary to take a few samples, possibly 20 would be sufficient, to determine the approximate ground cover. Using Table III, it should then be possible to determine how many more samples are required, correcting at a later stage when more samples have been taken, if this is considered necessary.

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An Examination of some Symbiotic Systems for Fixation of Nitrogen

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With one Figure in the Text

ABSTRACT

By the use of ^{15}N the occurrence of fixation of elemental nitrogen has been demonstrated in the lichens *Collema granosum* and *Leptogium lichenoides* (both of which contain *Nostoc* as a symbiont), and also in the liverwort *Blasia pusilla* (which has *Nostoc*-containing cavities in the thallus). The circumstances indicate that the fixation in these instances should be attributed to the *Nostoc*.

No evidence of fixation was obtained in similar isotopic tests on the mycorrhizal roots of intact plants of *Calluna vulgaris* and of *Pinus sylvestris*, although these, more especially in the case of *Calluna*, have been held by some previous authors to be nitrogen-fixing.

INTRODUCTION

EXAMPLES of symbioses[1] with which fixation of nitrogen is associated are provided by nodulated legumes and by certain non-legume plants which also bear root nodules (Bond, Fletcher, and Ferguson, 1954). From time to time the suggestion has been made that fixation might be associated with some other symbiotic associations. The isotopic nitrogen technique, used by Wilson and his collaborators (Burris, Eppling, Wahlin, and Wilson, 1943; Wilson, 1952) for the study of fixation by leguminous plants and various free-living organisms, provides a reliable (though expensive) method by means of which even slight fixation can readily be detected in short-term experiments. This technique has here been employed in an examination of four symbiotic systems for nitrogen fixation, namely:

1. the lichen symbiosis, the examples chosen being *Collema granosum* (Scop.) Schaer. and *Leptogium lichenoides* (L.) Zahlbr., both of which include *Nostoc* as one of the symbionts;
2. *Blasia pusilla* L., a liverwort with cavities containing *Nostoc* in the thallus;
3. *Calluna vulgaris* (L.) Hull, the roots of which regularly show a mycorrhizal association with a fungus; and
4. *Pinus sylvestris* L., where there is again a fairly regular mycorrhizal condition.

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[1] As used in this paper the term symbiosis does not necessarily imply anything more than association of organisms.

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Following on the demonstration of the nitrogen-fixing power of certain blue-green algae in pure culture (see Wilson and Burris, 1953, and Fogg, 1953, for reviews of the literature), several authors have surmised that fixation might also be associated with symbiotic systems involving blue-green algae. Henriksson (1951) showed that a strain of *Nostoc* isolated from the lichen *Collema tenax* fixed nitrogen in pure culture, but there appear to have been no previous tests for fixation carried out on an actual lichen. Garjeanne (1930) cultured *Blasia* on sand moistened with a mineral solution free of combined nitrogen, certain cultures being inoculated with *Nostoc* while others remained uninoculated. Growth was similarly poor in both sets of cultures, indicating that the *Nostoc* cavities conferred no benefit on the *Blasia*, although the possibility of nitrogen fixation having occurred in the cavities does not seem to be excluded. In another experiment the two types of *Blasia* were again cultured, combined nitrogen being this time supplied. The percentage nitrogen contents of the two samples were found to be practically identical, but Garjeanne gives reasons for regarding this result with reserve. A further point is that the provision of combined nitrogen would tend to depress any fixation by the *Nostoc* (Fogg, 1953). Garjeanne also points out that in general the *Blasia* grew rather poorly in sand culture, and that his findings would not necessarily hold for more vigorous field material.

Rayner (1922) reported that young plants of *Calluna vulgaris*, inoculated with *Phoma radicis Callunae*, continued to grow healthily in tube culture on a medium free of combined nitrogen in amounts detectable by Kjeldahl analysis, though no details of this analysis were given and no control plants were available to prove that the rooting medium could not provide the traces of combined nitrogen required by such small plants. Neilson Jones and Smith (1928) submitted four of the plants grown previously by Rayner on an agar medium to Kjeldahl analysis, the mean nitrogen content per plant being found to exceed that of a *Calluna* seed by 0·012 mg., ascribed to fixation. This finding is invalidated by the fact that the plants had been preserved in alcohol prior to analysis. The same authors described careful tests on cultures of the above *Phoma* isolate, with results which suggested a slight capacity for fixation, amounting to a fraction of a milligramme per culture. Fixation had previously been claimed by Ternetz for strains of *Phoma radicis* isolated from other ericaceous mycorrhiza, this work and also that of Duggar and Davis, who reported fixation in *Phoma betae*, being discussed by Neilson Jones and Smith (loc. cit.). Wilson (1952) reports negative results for fixation by *Phoma casuarinae*, by isotopic technique. It should be noted that Harley (1950) concluded that final proof is lacking that the typical endophyte in Calluna mycorrhiza is *Phoma radicis*.

The position regarding *Calluna* mycorrhiza is thus confused, and in view of the ecological importance of this plant it is desirable that its status in relation to nitrogen fixation should be clarified.

The surveys of the literature provided by Rayner (1927), Hatch (1937), and Björkman (1949) show that though several investigators have considered the possibility of nitrogen fixation by the mycorrhizal roots of *Pinus*, such studies as have been made of the ability of inoculated plants to grow in nitrogen-poor media have provided no support for the idea; nevertheless in view of the interest to forestry an isotopic examination was considered desirable.

METHODS

Origin of the plant material

Material of *Collema granosum* was collected from the surface of calcareous rock, some of it shortly before the test, while the remainder had been collected 8 weeks earlier and maintained in aerated culture vessels supplied with a nitrogen-free mineral solution. *Leptogium lichenoides* was collected from the Loch Lomond mica schist a few days before required. Field material was also employed of *Blasia pusilla*. Numerous *Nostoc* cavities were present on this material, and in section showed the normal appearance as figured, for example, by Garjeanne (1930).

Young plants of *Calluna vulgaris* were obtained from Dr. L. W. Poel of St. Andrews University. They had been raised from seed in the greenhouse, and at the time of transference to Glasgow were growing in a medium (pH 4·6) consisting of peat collected from a *Calluna* area and sand. The plants remained undisturbed in this medium until shortly before the test; they were of healthy appearance, and microscopic examination revealed a typical mycorrhizal condition in many of the roots as a result of natural infection.

Plants of *Pinus sylvestris* were raised in the greenhouse from seed provided by the Forestry Commission, 6 weeks' old seedlings being transplanted into a medium (pH 3·8) comprising equal parts of sand, peat, and a top-soil collected from a pine wood. The plants subsequently showed vigorous growth and a profuse development of typically forked mycorrhizal rootlets. Data provided later show that the fresh weight of these rootlets amounted to about 8·5 per cent. of the weight of the whole plant, a figure which, incidentally, is of the same order as the corresponding values for the nodules of root-nodule plants (Ferguson and Bond, 1953).

Enclosure of material for fixation test

With the lichens and *Blasia*, immediately prior to the fixation test weighed samples of thallus, brushed free from soil, were placed in glass specimen tubes of 15-ml. capacity, each closed by a rubber stopper bearing a capillary connexion. Five millilitres of sand had been placed previously in each tube and moistened with a nitrogen-free mineral solution.

In the case of *Calluna* and *Pinus* the root systems only were enclosed. Two to three weeks before the test, plants were transplanted singly into wide-necked bottles of 50-ml. capacity, containing a similar rooting medium to that in which the plants had been growing previously, and each fitted with a rubber stopper bearing a capillary tube and bored with a wide hole through which the shoot of the plant was passed. Subsequently a split rubber ring was pushed down into the hole in the stopper, forming a base for the 'plasticine' used to seal the plant in for the isotope test (Fig. 1). The actual gas space in each bottle fitted up as described was of the order of 20 ml.

Exposure to ^{15}N and subsequent analysis

Gaseous nitrogen containing excess ^{15}N was prepared in a nitrometer by the action of sodium hypobromite on ammonium nitrate containing 36·2 atom per cent. ^{15}N in the ammonium radical. The nitrogen was transferred to a gas burette and thoroughly shaken with 5 per cent. sulphuric acid as a precaution against the possible presence of traces of ammonia containing excess ^{15}N. (*Note*: The fact that, as will be shown later, in certain experiments no increase in ^{15}N content was shown by plants exposed to the isotopic gas mixture proves that no combined ^{15}N assimilable by plants was present.)

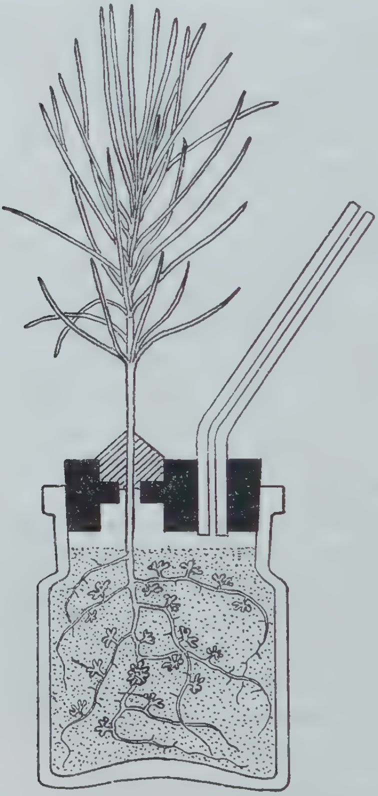

FIG. 1. Arrangement used for enclosure of root systems of *Calluna* and *Pinus*. See text. ($\times \frac{2}{3}$.)

The specimen tubes containing the thalloid material, or the bottles with sealed-in *Calluna* or *Pinus* plants, were attached to a manifold built from capillary tubing, with stations for six plant containers and connexions to a manometer, a filter-pump, and the gas burette containing the nitrogen, the whole system being erected in the greenhouse. After a thorough test for gas-tightness the system was evacuated to 0·5 atmosphere, and the special nitrogen then admitted until the pressure was restored to 1 atmosphere. The apparatus was now occupied in respect of the gas phase by a mixture composed approximately of 10 per cent. oxygen and 90 per cent. nitrogen, the latter containing 20 atom per cent. of ^{15}N (confirmed by mass spectrometer analysis). In the tests with the lichens and *Blasia*, in order to provide for photosynthesis carbon dioxide was added to the gas mixture to give a final proportion of 1·5 to 2·5 per cent. in different experiments, with corresponding reduction in the proportion of nitrogen.

Suitably placed stopcocks made it possible to remove the plant containers from the manifold, after an interval of some hours to permit of equilibration. The containers were left sealed up for several days under greenhouse conditions, after which the plant material was removed and submitted to the Kjeldahl process. No obvious signs of deterioration in the plant material as a result of the enclosure were observed. The distillates from the Kjeldahl process, after titration of aliquots to permit of the estimation of total nitrogen, were evaporated down to a small volume and subsequently assayed for ^{15}N content by the staff of the Chemistry Department of this University, a Metropolitan-Vickers Mass Spectrometer II being used.

Control samples were prepared from similar plant material which had not been exposed to the isotopic gas mixture. Numerous analyses by previous investigators have shown the normal ^{15}N content of the nitrogen in biological and other material to be 0·368 atom per cent. Thus the main reason for the examination of control samples is as a check on the analytical procedure. The mean of the eleven control values reported in this paper is 0·376 atom per cent., the range being 0·363 to 0·386 and the standard deviation 0·008. Thus a mass spectrometer value exceeding 0·400 can be accepted as satisfactory evidence that the sample concerned has undergone enrichment with ^{15}N above the normal level.

DATA OBTAINED

The results of the tests with *Collema* and *Leptogium* are presented in Table I. Four of the five samples of *Collema* exposed to the isotopic nitrogen show a significant increase in ^{15}N content, indicative of fixation. The increases with *Leptogium* are larger and more consistent. The total nitrogen contents of the samples being known (see table), calculations can be made which indicate that the mean total fixation (^{14}N and ^{15}N) during the period of the test must have amounted to approximately 0·08 mg. per sample in the case of *Leptogium*.

Data obtained with *Blasia* are provided in Table II. A considerable increase in ^{15}N content is shown by each of the samples exposed to the isotope, providing clear evidence of fixation. Calculation as above indicates a fixation of 0·03 mg. nitrogen per sample.

The results yielded by *Calluna* are given in Table III. The root systems

TABLE I

Tests for Fixation by Collema granosum and Leptogium lichenoides

	Sample number.	^{15}N content of thallus, atom per cent.	
		*Collema.**	*Leptogium.†*
Exposed to excess ^{15}N	1	0·397	0·583
	2	0·408	0·597
	3	0·406	0·487
	4	0·454	0·589
	5	0·447	0·506
	6	—	0·567
Controls	7	0·377	0·371
	8	—	0·373

* Exposure to excess ^{15}N commenced July 15 and continued for 5 days. Mean temperature during period 19° C. Fresh weight per sample 2·5 g., mean total nitrogen content 3·5 mg.

† Exposure to excess ^{15}N commenced Sept. 22 and continued for 7 days. Mean temperature 21° C. Fresh weight per sample 1·2 g., mean total nitrogen content 8·9 mg.

TABLE II

*Test for Fixation by Blasia pusilla**

	Sample number.	^{15}N content of thallus, atom per cent.
Exposed to excess ^{15}N	1	0·568
	2	0·592
	3	0·633
	4	0·541
	5	0·612
	6	0·754
Controls	7	0·379
	8	0·386

* Exposure to excess ^{15}N commenced Sept. 24 and continued for 7 days. Mean temperature 19° C. Fresh weight per sample 1·2 g., mean total nitrogen content 2·7 mg.

TABLE III

*Test for Fixation by Calluna vulgaris**

	Plant number.	^{15}N content, atom per cent.	
		Root system.	Shoot.
Root systems exposed to excess ^{15}N	1 & 2	0·387	0·379
	3 & 4	0·378	0·376
	5 & 6	0·383	0·398
Controls	7 & 8	0·386	0·376

* Exposure to excess ^{15}N commenced Sept. 21 and continued for 8 days. Mean temperature 21° C. Age of plants 18 months; mean height of shoot 10 cm.; mean fresh weight of root system 0·28 g., and of shoot 0·24 g.

TABLE IV

*Test for Fixation by Pinus sylvestris**

	Plant number.	^{15}N content of plant as a whole, atom per cent.
Root systems exposed to excess ^{15}N	1	0·382
	2	0·370
	3	0·383
	4	0·369
	5	0·369
Controls	6	0·385
	7	0·373

* Exposure to excess ^{15}N commenced Aug. 18 and continued for 7 days. Mean temperature 21° C. Age of plants 19 weeks; mean height of shoot 7 cm.; mean fresh weight of shoot 0·44 g., and of root system 0·27 g.; mean fresh weight of mycorrhizal roots alone, per plant, 0·06 g.; mean total nitrogen content per plant 4·0 mg.

TABLE V

*Further Test for Fixation by Pinus sylvestris**

	Plant number.	^{15}N content, atom per cent.	
		Root system.	Shoot.
Root systems exposed to excess ^{15}N	1 & 2	0·376	0·361
	3 & 4	0·380	0·379
	5 & 6	0·388	0·379

* Exposure to excess ^{15}N commenced Aug. 26 and continued for 6 days. Mean temperature 21° C. In size, &c., the plants were similar to those used in the preceding experiment.

TABLE VI

*Results of comparable Test with Nodulated Plants of Alnus glutinosa**

	Plant number.	^{15}N content, atom per cent.	
		Root system including nodules.	Shoot.
Root systems exposed to excess ^{15}N	1	0·557	0·481
	2	0·757	0·611
Control	3	0·363	0·367

* Exposure to excess ^{15}N commenced Aug. 27 and continued for 5 days. Mean temperature 20° C. Mean height of shoot 8 cm.; mean fresh weight of shoot 0·70 g., and of root system 0·40 g.; mean total nitrogen content per plant 6·4 mg.

were analysed separately from the shoots in order to increase the prospects of detecting slight fixation by the mycorrhiza. Actually there is no significant increase in ^{15}N content in any of the samples, and thus no evidence of fixation. The same applies to the results obtained with *Pinus* which are detailed in Tables IV and V, the roots and shoots being analysed separately in the second experiment.

DISCUSSION

The data show that fixation of free nitrogen was associated with both the lichens that were tested. Since aseptic conditions were not provided in the present study, it might be suggested that there was superficial contamination with nitrogen-fixing organisms such as *Azotobacter*, and that the fixation was due to these rather than to the *Nostoc* constituent of the lichen itself. Actually it is impossible to conceive of sufficient contamination having been present. For example, the data for *Azotobacter* provided by Burk (1930) show that for that organism to have been wholly responsible for the fixation of 0·08 mg. nitrogen associated with each sample of *Leptogium*, at least 8 mg. of glucose or an equivalent amount of alternative substrate would have had to be available. It is difficult to suppose that such relatively large amounts of metabolic materials escaped from the surface of the lichen thallus, and it is concluded that any superficial *Azotobacter* present played no major part in the observed fixation.

Several authors have reported the isolation of *Azotobacter* from the interior of the thallus of various lichens, though others have been unable to confirm this (see Abbayes, 1951, for a review of this question). For any such symbiotic *Azotobacter* to have contributed significantly to the observed fixation, the organism would have to be present in amounts which should be detectable microscopically. Thus if it is assumed for the moment that symbiotic *Azotobacter* was responsible for all the fixation observed in *Leptogium*, then again from the data of Burk (loc. cit.) the fixation of 0·08 mg. nitrogen should be accompanied by the formation of 0·8 mg. of new *Azotobacter* dry cell substance, additional to that present at the commencement. The mean dry weight per sample of *Leptogium* being 284 mg., it follows that *Azobacter* cells should make up at least 0·3 per cent. of the substance of the lichen thallus, and probably a good deal more. Careful microscopic study of sections of this lichen provides no evidence of the presence of *Azotobacter* to such an extent. It is concluded that the fixation should be attributed to the *Nostoc* constituent. The data obtained provide no information on how the fixed nitrogen is distributed as between the algal and fungal partners of the lichen.

In respect of *Blasia* it is again difficult to contemplate superficial contaminants having been wholly or mainly responsible for the observed fixation, and the most reasonable course is to ascribe it to the *Nostoc*. The data obtained have been closely examined in relation to the further question as to whether part of the nitrogen fixed in the *Nostoc* cavities becomes available to the *Blasia* itself. Careful measurements on a typical portion of thallus, as used in the fixation test, showed that the combined volumes of the cavities, which are individually very minute, amounted only to 1·1 per cent. of the total volume of the thallus. The weight relationship must be of the same order. The proportion of nitrogen in the make-up of the cavities is, however, no doubt greater than in the case of the thallus tissues. If it is assumed to be 50 per cent. greater, and if it is also assumed that at the end of the test period the

^{15}N content of the nitrogen of the cavities had attained the high level of 10 atom per cent.,[1] then mass spectrometer values based on the examination of the thallus as a whole could not attain the level of most of those shown in Table II unless additional fixed ^{15}N had passed from the cavities into the thallus. Hence it is probable but not certain that such transfer of fixed nitrogen occurred. The behaviour of *Nostoc* in pure culture, where a considerable part of the fixation products is excreted into the medium (Fogg, 1953), suggests that transfer is likely to occur in the symbiotic association.

In connexion with the tests on *Calluna* and *Pinus*, it has to be considered whether any feature of the experimental treatment could have suppressed any nitrogen-fixing potentialities and led to the negative results actually obtained. One possible criticism is that transplanting was carried out not very long before the tests. This was done carefully and with no visible detriment to the plants, but the functioning of the mycorrhiza may be adversely affected by a partial severance of the hyphal connexions with soil mycelium. It is, however, difficult to suppose that a total suppression of fixatory activity would ensue.

Another point to be considered is the oxygen supply for the root systems during the tests. As noted, the method by which the isotopic nitrogen was introduced to the plant containers, though convenient and advantageous in avoiding exposure of the plant material to complete evacuation, provided only 10 per cent. of oxygen initially in the gas mixture supplied to the roots, or in absolute terms, 2 ml. per root system. Evidence that this somewhat reduced oxygen supply does not inhibit other nitrogen-fixing systems is provided by the positive results obtained with lichens and *Blasia* and by many other such results gained with non-legume root-nodule plants, to be published separately. Detailed reference will be made here, however, to one test with nodulated alder plants. These had been raised in sand and then transplanted into soil similar to that used for *Pinus*. The root systems were enclosed and exposed to isotopic nitrogen by exactly the same procedure as was used for *Pinus* and *Calluna*. The results are shown in Table VI. Substantial enrichment with ^{15}N, indicative of that occurrence of fixation, is shown by the plants, confirming that the experimental conditions were sufficiently favourable for the exhibition of fixatory powers where these were inherent in the material.

Though tests with other material of *Calluna* and *Pinus* of different origin are perhaps desirable before a final conclusion is reached, the evidence now presented permits the conclusion that there is at least no regular association of any significant fixation of nitrogen with the mycorrhizal roots of these two plants.

SUMMARY

Isotopic nitrogen (^{15}N) has been employed in an examination of several symbiotic systems for fixation of nitrogen.

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[1] Burris et al. (1943) reported that a species of *Nostoc*, exposed to 28 atom per cent. excess ^{15}N for 8 days, accumulated 8 atom per cent. ^{15}N in its cells.

Evidence of the occurrence of fixation has been obtained in respect of the lichens *Collema granosum* and *Leptogium lichenoides*, both of which contain the blue-green alga *Nostoc* as one of the symbionts. Positive evidence of fixation was also obtained with the liverwort *Blasia pusilla*, which has cavities containing *Nostoc* in the thallus. Field material was employed in the tests with these three species. The circumstances indicate that the fixation should be attributed to the *Nostoc*. The data obtained in the case of *Blasia* suggest but do not prove that part of the fixed nitrogen was transferred from the *Nostoc* cavities to the thallus of the liverwort.

Negative results for fixation were obtained in tests on the mycorrhizal roots of intact plants of *Calluna vulgaris*, a finding which is contrary to the view held by some previous authors. Negative results were also obtained with the mycorrhizal roots of *Pinus sylvestris*, in agreement with the findings of previous investigators using less sensitive methods. The plants employed of these two species were raised in the greenhouse, the mycorrhizal condition being set up by natural infection from the rooting medium provided.

ACKNOWLEDGEMENTS

In addition to acknowledgements already made, the authors wish to thank Dr. R. I. Reed and Mr. A. Macdonald, of the Department of Chemistry of this University, for the mass spectrometer analyses, and Dr. James Bell of the same Department for suggestions during the course of the work. Mr. A. C. Crundwell kindly provided the material of *Blasia pusilla* and gave information regarding that species. One of us (G. D. S.) held a Carnegie Research Scholarship during the investigation.

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The Occurrence of Translocated Antibiotics in Expressed Plant Sap

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With two Figures in the Text

ABSTRACT

The details of a convenient laboratory press are presented and a procedure by which plant sap is readily obtained is described. The pressing procedure has been shown to release 53–74 per cent. of the total plant water as expressed sap.

Preliminary observations on the uptake and translocation of antibiotics by higher plants obtained by examining expressed sap are described. It is shown that the assay of expressed sap provides a measure of the griseofulvin and chloramphenicol concentration in leaves of plants grown in solutions of these antibiotics that does not differ significantly from the value obtained by the assay of water extracts.

Extraction of leaves from plants grown in griseofulvin solutions with organic solvents demonstrates a much greater antibiotic content than is indicated by the assay of expressed sap. To explain this difference a 'free' antibiotic fraction, obtainable in expressed sap or water extracts, and a 'bound' fraction recovered from the leaf only by organic solvent extraction are postulated.

INTRODUCTION

THE extensive use that has been made of plant sap in studies of the movement of solutes into and within higher plants has been reviewed by Broyer (1939). Sap has been expressed from both living tissue and from tissue that has been killed by various physical and chemical treatments. Sap expression from living tissue, which requires high pressures and special precautions to avoid shearing forces, normally yields only a small proportion of the total liquid present. This fraction may be low in solute concentration. Larger proportions of the total fluid containing a higher percentage of solutes are released from tissue that has been killed prior to pressing.

Phillis and Mason (1937) and Bennet-Clark and Bexon (1939) considered sap expressed from living tissue to be derived from the vacuole and sap obtained from killed tissue to be cytoplasmic fluid; Broyer and Hoagland (1940) concluded that no such clear distinction could be made since the quantity of fluid and the concentration of solutes appeared to be a function of the pressure and pressing time. They considered that the most satisfactory procedures for study of absorption and accumulation of solutes in

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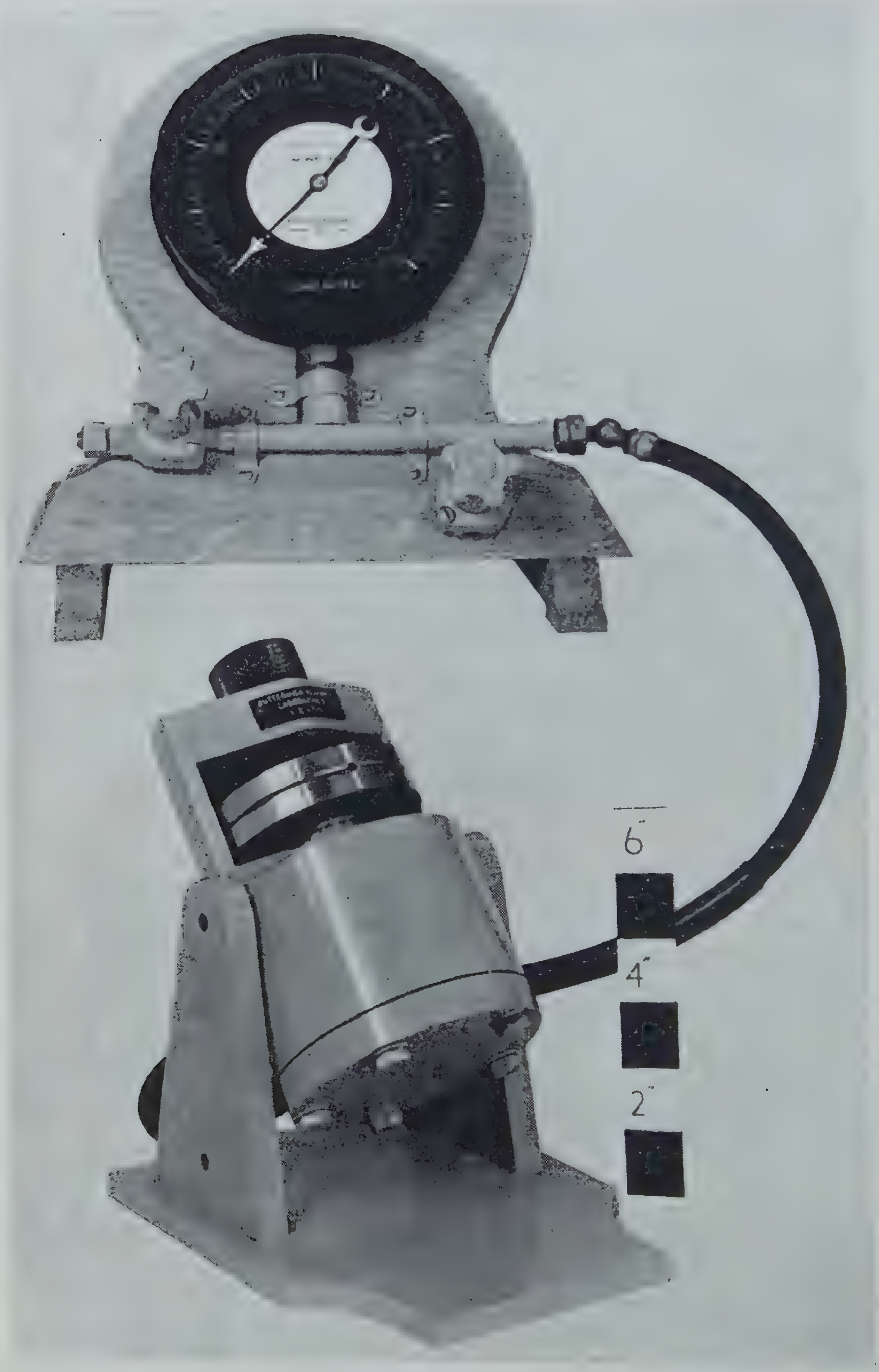

Fig. 1. The plant press.

complex tissues are 'those which give the largest yields of expressible fluid'.

The present communication provides details of a convenient laboratory press, describes a procedure by which plant sap is readily obtained, and presents preliminary observations on the absorption and translocation of antibiotics by higher plants obtained from examining expressed sap.

THE PRESS

For the present use a press capable of handling small quantities of whole tissue with a minimum of shearing action and a minimum of retention of the expressed sap was required. This was achieved with the press illustrated in Figs. 1 and 2.

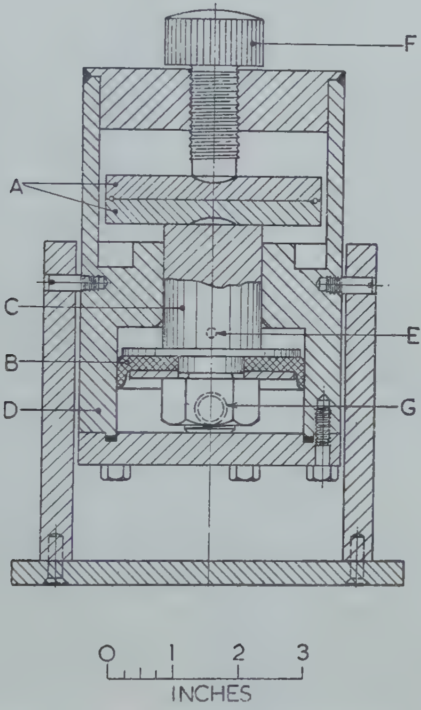

FIG. 2. *Section through press*: A, press plates; B, rubber cup-ring; C, piston; D, cylinder; E, exit hole for air above piston; F, locating screw; G, entrance hole for high-pressure air.

Compressed air, from service mains with a maximum available pressure of 60 lb. per sq. in., drives the piston C which presses the two plates A together. These plates, which are identical, are made of stainless steel $5\frac{5}{16}$ in. in diameter and $\frac{3}{8}$ in. thick, their working faces are ground and lapped flat and have a narrow annular groove at the edge. The plates can be removed from the press for inserting the tissue, which is usually one or two leaves. The expressed sap is collected in the groove and delivered through a notch in the edge of the plate. The reverse of each plate is recessed to allow location with screw F. This screw is tightened lightly to take the thrust of the piston.

The piston is sealed in the cylinder D with a rubber cup-ring B. Compressed air enters the cylinder through hole G. The air is admitted through an $\frac{1}{8}$-in. 'Saunders' stop valve until the required loading is obtained; a recalibrated pressure gauge (6 in. dial. 0 to 60 p.s.i.) indicates the total thrust in pounds. A second valve releases the pressure.

The press is supported on brass pivots in a steel stand. In use it is tilted over a small beaker which collects the sap dripping off the plates.

PRESSING PROCEDURE

Killing of plant material prior to pressing allows the expression of a large proportion of the tissue fluid with little pressure. The method of killing, whether by cold, heat, or organic solvents, is largely a matter of convenience.

In most studies plant tissue has been frozen, thawed, and then pressed. This procedure, however, is slow, and in the case of broad bean (*Vicia faba*) there was extensive blackening before the material had thawed sufficiently for pressing.

Killing of tissue by immersion in boiling water was satisfactory in work with griseofulvin, which is thermostable, but, as will be shown by data to be presented later, moisture retained by tissue immersed in boiling water appreciably dilutes the sap recovered.

Tissues are also killed rapidly by immersion in organic solvents (Chibnall, 1923), and killing by immersion in ether for 30 seconds has been used with three antibiotics. This was the only method used for the comparatively thermolabile antibiotics chloramphenicol and streptomycin. After killing, plant tissue was placed on filter-paper to remove as much as possible of the excess killing material and then subjected to a pressure of approximately 300 lb./in.^{2} After pressing, ether collected with the expressed sap was reduced to a non-toxic level by evaporation at laboratory temperatures under reduced pressure. The plant saps obtained were clear though coloured and antibiotics could be assayed without further treatment.

Table I illustrates the efficiency of the pressing procedure when applied to a variety of plants and also serves to demonstrate the relative value of pretreatment with ether. The amount of fluid expressed was measured by weighing the leaves before and after pressing and the total water content was derived from fresh and dry weights of the tissue. An approximate estimate of the loss of expressed sap due to retention by the press was obtained by measuring the volume of fluid collected and comparing that value with the weight lost by the tissue in being pressed.

The pressing procedure has been found to release 50–80 per cent. of the total plant water as available sap. The design of the press is such that when leaves were pretreated with ether approximately one-half of the expressed sap was collected. The greater proportion of the expressed sap which was collected when the leaves were killed by boiling water is probably due to dilution of the sap by moisture retained by the leaves from the killing-bath. In general terms, a single bean leaf would yield approximately 0·7 ml. of sap and samples large enough for assay were obtained by pressing 3 or 4 leaves.

TABLE I

Percentage of Total Water expressed and recovered from Killed Leaves

Plant.	No. replicates.	Killing agent.	Percentage of total water expressed as sap.	Percentage of expressed sap collected.
Broad bean	10	Ether	74	56
	5	Boiling water	72	104
Tomato	5	Ether	53	55
	5	Boiling water	63	97
Lettuce	6	Ether	67	50
	6	Boiling water	61	77
Oat	9	Ether	64	—

APPLICATION OF THE PRESSING PROCEDURE TO STUDIES OF THE ABSORPTION AND TRANSLOCATION OF ANTIBIOTICS

Materials and methods

Observations were made on the translocation of griseofulvin, chloramphenicol, and streptomycin.

Plants were grown with their roots in nutrient solutions containing the antibiotics. In preliminary trials plants were treated for 10–14 days under greenhouse conditions before the leaves were tested. In later studies plants were treated for 18 hours in a chamber at a temperature of 25° C. and a relative humidity of about 60 per cent.

TABLE II

The Effect of Expressed Leaf Sap on Antibiotic Assays in the Absence of Yeast Extract in Lab-Lemco Agar

	Apparent percentage loss of activity in the presence of plant sap	
Plant.	Streptomycin.	Chloramphenicol.
Broad bean	80	70
Tomato	0	30
Lettuce	0	40
Cabbage	0	40

Griseofulvin was assayed by observing the effect on germ-tubes of *Botryiis allii* (Brian, Curtis, and Hemming, 1946). Assays with measured quantities of griseofulvin in expressed sap gave results identical with those in which no cell sap had been included; thus no corrections were required.

Chloramphenicol and streptomycin were assayed by the cylinder-plate technique, using dehydrated Lab-Lemco agar supplemented with 0·3 per cent. yeast extract and seeded with a *Bacillus subtilis* (N.C.T.C. 7241) spore suspension. Tests with measured quantities of these antibiotics added to plant saps showed that omission of the yeast extract led to considerable variation in zone size as compared with the standards. This is illustrated in Table II. This apparent loss is eliminated completely by the addition of yeast extract to the agar and by the additional precaution of adding at least an equal volume of distilled water to the sap prior to assay.

Water extracts of plant tissues were prepared by trituration of the material with distilled water. From the weight of tissue extracted, the volume of water used for extraction, and the antibiotic activity of the extract, the antibiotic content of the plant tissue was calculated.

The chloroform extraction of griseofulvin and its estimation will be described in a later publication.

Results and discussion

Table III illustrates the antibiotic activity shown by the expressed sap of plants which were grown in the greenhouse for 10–14 days in solutions containing 25 μg./ml. of the antibiotics. Assays of chloramphenicol and streptomycin were carried out on agar not fortified by yeast extract and are, therefore, subject to the errors illustrated in Table II. In spite of the errors, however, the table does illustrate that these antibiotics can be recovered in the expressed sap of a range of plants. The absence of griseofulvin activity in the expressed sap of lettuce was unexpected since this compound has previously been demonstrated in water extracts of leaves of lettuce grown in antibiotic solution (Brian, Wright, Stubbs, and Way, 1951). Failure to demonstrate streptomycin in the expressed sap as well as water extracts of broad bean plants grown in solutions containing low concentrations of the antibiotic has been confirmed by repeated experiments.

TABLE III

Concentration of Antiobiotic assayed in Expressed Leaf Sap of Plants treated for 10–14 Days with 25 μg./ml.

Plant.	No. replicates.	Mean concentration of antibiotic μg./ml.		
		Griseofulvin.	Chloramphenicol.	Streptomycin.
Broad bean . .	5	0·3	20	0
Tomato . .	5	0·6*	11	150
Lettuce . .	5	0	5	31
Cabbage . .	5	0·5	4	5
Wheat . . .	5	0·4*	5	19

* Mean of 3 replicates.

A comparison of the antibiotic concentration calculated from fresh weights, in leaves of treated broad bean plants as determined by the assay of various leaf preparations, is presented in Table IV. The plants were treated in a constant temperature and humidity chamber for 18 hours, after which the leaves were collected.

The griseofulvin concentration in the leaf tissue shown by assay of the expressed sap (3µg./g.) does not differ significantly from that obtained from the water extract of the leaf (4µg./g.). However, both are markedly lower than the value obtained from a chloroform extract of a parallel series of plants (15µg./g.) and from the chloroform extract of pressed residues (31µg./g. pressed residue). This discrepancy may be explained by assuming that the concentration in the expressed sap is a true measure of the griseofulvin in solution in the tissue and that the remainder is bound in some water-insoluble form. It is possible to test this assumption by calculating the total extractable griseofulvin concentration in the pressed leaves and comparing the result with the antibiotic concentration found in unpressed leaves. This calculation yields a value of 13µg./g. which is virtually the same as that obtained by extraction of unpressed leaves (15µg./g.). Therefore the assay of expressed fluid yields a true estimate of the amount of griseofulvin present in the leaf sap and pressing may distinguish between two forms of griseofulvin in the tissue.

TABLE IV

Concentration of Antibiotic in Leaves of Treated Broad Bean Plants determined by the Assay of Various Leaf Preparations

Antibiotic.	Nature of preparation assayed.	Concentration of antibiotic µg./g. of leaf tissue.*			
		Concentration of antibiotic in solution µg./ml.			
		25.	50.	100.	500.
Griseofulvin	Expressed sap	3	—	—	—
	Water extract	4	—	—	—
	CHCl$_3$ extract	15	—	—	—
Chloramphenicol	Expressed sap	—	19	—	220
	Water extract	—	20	—	200
Streptomycin	Expressed sap	—	—	5	273
	Water extract	—	—	0	136

* Mean of 6 replicates.

Assay of expressed sap from plants grown in solutions of chloramphenicol has been found to provide a measure of the antibiotic concentration in the leaf that does not differ significantly from that obtained by assay of a water extract of the tissue. The concentration of antibiotic in the leaf was found to vary directly with the concentration in solution. Though not shown in Table IV, this relationship has been found to exist for plants raised in griseofulvin solutions.

The assay of sap from plants grown in solutions containing streptomycin demonstrates a greater concentration of antibiotic in the leaf than is indicated by assay of water extracts of the tissue. This difference may result from the cationic nature of streptomycin. An increased concentration of streptomycin applied to the roots of broad bean plants resulted in an increase in the concentration found in the leaves. No extraction of leaf tissue of plants grown in chloramphenicol or streptomycin solutions was attempted with organic solvents.

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Studies in Vernalisation of Cereals

XIII. Photoperiodic Control of Stages in Flowering between Initiation and Ear Formation in Vernalised and Unvernalised Petkus Winter Rye[1]

BY

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With 7 Figures in the Text

ABSTRACT

The photoperiodic reactions of spring rye and vernalised and unvernalised winter rye have been studied in detail and interpreted in terms of a schema presented in earlier papers. Vernalisation treatments ranging from 0 to 13 weeks were used and three daylengths (continuous light, C.L.; normal days, L.D.; 10-hr. days, S.D.), and the interaction effects of these factors were studied on the following developmental features: time to initiation of flowering and to 'double-ridge' formation; progress of differentiation and growth rate of the apex of the main shoot before and after initiation. Systematic dissections of the apex were carried out to obtain data for this study. The validity of the concept of 'minimal leaf number' was re-examined and confirmed by using dwarf embryos with subnormal numbers of primordia at germination. Unvernalised winter rye was shown to behave as a short-day plant before flower initiation since photoperiodic induction by short days is annulled by interrupting the dark period by a short light period at low intensity. The effect of exposure of a single leaf to continuous light, while the remainder of the plant was kept in short days, has been shown to be transmitted to the apex and hastens both flower induction and spikelet development in fully vernalised rye. Vernalised winter rye and spring rye thus behave as long-day plants.

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[1] The paper incorporates data from a Ph.D. thesis of University of London submitted by Mrs. M. B. Gott.

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INTRODUCTION

EARLIER papers in this series have dealt with the factors concerned in the transition from the vegetative to reproductive phases in the development of the rye plant. This transition involves the whole apical meristem, with the formation of a terminal inflorescence and cessation of further production of vegetative organs.

In the mature grain of rye four primordia are already present at the apical growing-point of the embryo: of these, three are recognizably foliar, while the fourth is present as a simple ridge. As new primordia develop the terminal meristem elongates to accommodate an increasing number of simple ridges, which, at that stage, give no indication of their future status as leaf or flower. Later each leaf develops in its axil a bud which may grow into a branch or tiller. With the exception of a certain number of leaves below the ear, therefore, each primordium is a potentially double structure. In the relatively few leaves below the ear the axillary bud fails to appear, and in this region the internodes undergo rapid extension growth after flower initiation has begun, and constitute the culm. Under conditions favourable to flower initiation the simple ridges on the terminal meristem undergo a visible change. The tissue between the primordia swells so that each ridge becomes a double structure. The swollen tissue which may be regarded as the homologue of the 'tiller' buds of the lower leaves gives rise to the spikelets while the subtending bract is suppressed; in the mature ear it is absent, or at most represented in the lower spikelets of the ear by a minute ridge of tissue. The formation of these double ridges is the first perceptible sign of the termination of the vegetative phase.

In an earlier paper (Purvis and Gregory, 1937) it was shown that by varying daylength and temperature of germination the number of primordia producing either a leaf or a spikelet could be changed. These were referred to as 'labile primordia' and denoted 'physiologically double'. At that time it was thought that this range of labile primordia extended from the 7th to the 25th: all primordia below the 7th were supposed to be obligate leaves; all above the 25th obligate flower primordia. A maximum and a minimum leaf number was therefore postulated, and the stage of production of the minimal number of leaf primordia, preceding flower initiation, was regarded as the stage of 'ripeness to flower'.

The visible expression of the double nature of the 'labile primordia' referred to as 'double ridges' must not, however, be regarded as synonymous with the term 'physiologically double'; indeed, when 'double ridges' appear the floral nature of the initial is already determined and it is in general at this stage no longer labile. At one time it was surmised that *all* primordia above the 'minimal leaf number' belonged to the category of 'double ridges' and that under conditions unfavourable to flowering the spikelet primordium was inhibited whereas the 'bract' primordium subtending it grew into a foliage leaf. After flower initiation and the formation of 'double ridges' this possibility is realized, to which naturally occurring abnormalities in ear structure which have been described and can be artificially induced bear witness.

To establish the relations between flower initiation and the appearance of double ridges demanded a more extensive survey of the conditions at the apex using a wider range of daylength conditions and durations of vernalisation, and in this and forthcoming papers the results of further work will be presented. Particular attention has been directed to the re-examination of the concept of 'minimal leaf number'. The fact that in normal daylength the leaf number of rye may be reduced to 7 and very rarely 6, whereas in short days the number rises to above 20, suggested the possibility that in the complete absence of darkness the leaf number could be still further reduced, and that indeed flowering might occur without production of leaves beyond the four whose initials are already present in the embryo.

The use of continuous light for this purpose was indicated and an experiment was planned in 1940, but war conditions prohibited any such work which was therefore postponed until 1948. The flower-inductive effect of continuous light has long been known: thus Klebs had found (1913) that 96 hours of continuous exposure to light sufficed to induce the formation of flower primordia in *Sempervivum Funckii.* Cailachjan (1933) showed that winter cereals would flower in continuous light without vernalisation treatment. McKinney and Sando (1933, 1935) also reported accelerated heading of winter and spring cereals, both vernalised and unvernalised, by the use of continuous light.

EXPERIMENTAL METHODS

Throughout this work winter and spring rye (var. 'Petkus') was again used. In general the vernalisation technique and the cultural methods employed were those described by Purvis and Gregory (1937). Extensive periodical dissections were carried out on plants grown under different daylength régimes.

a. Light conditions

Continuous light was provided in 1948 by supplementing the natural summer day with light from tungsten filament lamps giving intensities at the plant level of 160–750 lux. In 1949 and 1950 a larger installation of forty-eight 60-Watt lamps provided an even illumination of 160–210 lux over an area of 30 × 20 feet. Both installations were situated in the open.

Long days were either unsupplemented summer days, or, in later experiments, uniformly of 17 hours' duration, in part of artificial light. At planting, in May, the period from sunrise to sunset was 14½ hours, rising to 16½ hours and falling to 13 hours at the end of the experiment in September. Photoperiodically effective light may have exceeded these periods by 1 hour or more, according to weather conditions.

Short days were in most experiments 10 hours' duration of natural daylight and 14 hours of complete darkness. Plants were darkened from 5 p.m. until 7 a.m. by three methods. (1) The plants, standing on trolleys, were moved into a ventilated double-walled shed (Purvis, 1934, Pl. XXI). (2) Groups of 9 pots were covered with cubical boxes (3-ft. edge) of duralumin metal, bedded into sand to exclude light. These covers were unventilated, and the high humidity encouraged rust and mildew. (3) Individual pots were covered with inverted pots of the same size. These were fitted with light-proof ventilation tubes and rubber fabric 'skirts' as shown in Fig. 1.

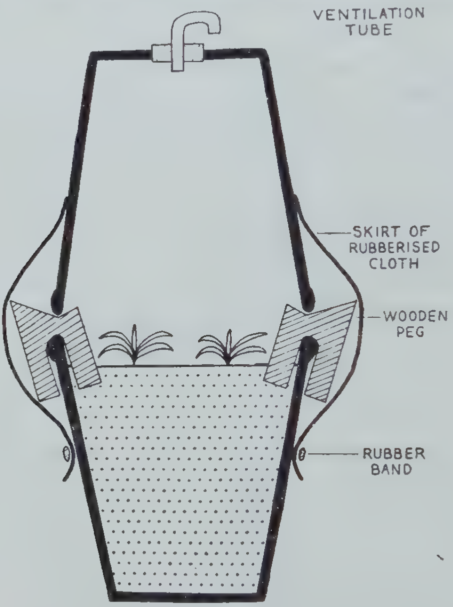

Fig. 1. Arrangement used for exposing plants to short days; for details see text.

Plants in continuous light and in long days received in addition to the supplementary artificial light from 4 to 7 hours more high intensity daylight than did those in 10-hour days.

b. Vernalisation treatment

The technique of checking growth during low-temperature treatment by limiting the moisture to 50 per cent. of the absolute dry weight was used, and the moisture content was maintained at the fixed level by the method finally developed by Purvis and Gregory (1952). Except where otherwise indicated, sterilization, imbibition, and vernalisation were carried out in darkness except for the short periods when the grain was being manipulated. Sterilization by calcium hypochlorite (Purvis and Gregory, 1952) was replaced by dusting with 'Spergon'.[1] This was rather more effective and simplified the preparation for vernalisation. Grain for the unvernalised series was also kept at 50 per cent. moisture content, but at 20° C., until it was judged to have reached the same stage of germination as the most highly vernalised grain. At the end of 13 weeks' vernalisation treatment only 5 lateral primordia were present at the apex, an increase of 1·5 on the number present before treatment.

c. Nutrition

Except where otherwise stated the plants were grown in sand culture as described by Purvis and Gregory (1937). In experiments not involving nitrogen as the variable factor studied, the nitrate applied was 1/10th that previously used. This had little effect on flowering, but tillering was checked, and manipulation of the plants thus facilitated (see pp. 93–95).

d. Temperature

Continuous temperature records showed small differences in temperature between the different daylength treatments. All methods of shortening the days raised the night minimum temperature; this was highest in the double-walled shed.

e. Dissections

Samples of about 12 plants were taken and the following data were recorded for the main shoot only: (1) Number of emerged leaves, including one immature leaf of which the lamina was not completely emerged. (2) Number of immature leaves within the sheath, including the last primordium which had grown enough to overlap the primordium situated immediately above it (Fig. 2). (1) and (2) together are recorded in the tables which follow as 'leaf number'. (3) The number of lateral appendages above this leaf, including both undifferentiated 'simple' ridges and also spikelet primordia if present. (4) The length of the apical meristem ('spike length'), measured from the last leaf, as defined above, to the tip. After leaf formation has ceased this becomes the ear length. (5) The progress of the meristem towards flowering, given as 'scoring' values. The basis on which these values are assigned to plants which have not flowered has already been given (Gregory and Purvis, 1938, and Purvis, 1947). It was not then made clear that for plants still in the vegetative phase these 'scores' were based on the 'spike length'. It had been observed that the first indication of double ridges usually occurs when the spike is about 1·6 mm. long. Assuming a linear relationship the advance towards this stage was assessed from the length of the spike. This relationship between spike length and maturity only holds

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[1] Tetra-chloro-parabenzoquinone, manufactured by U.S. Rubber Co., Naugatack, Conn., and kindly obtained for us by Mr. E. C. East.

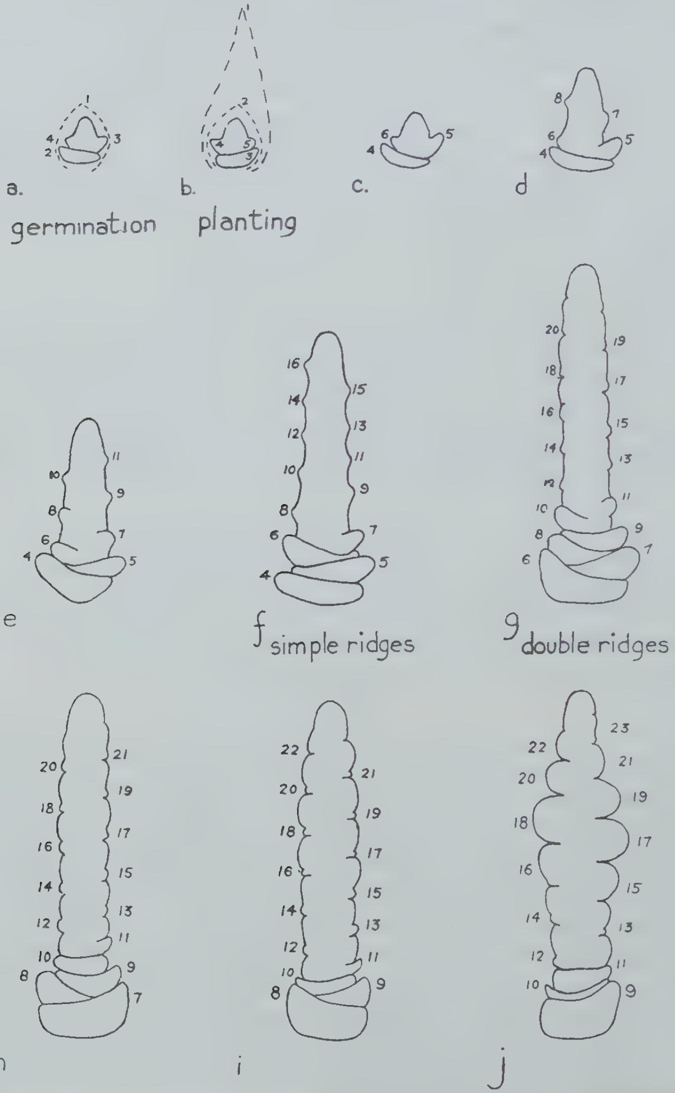

FIG. 2. Semidiagrammatic representation of apex of main shoot before and after flower initiation. The primordia are numbered in serial order.

good, however, under standard cultural conditions. The periodical dissections carried out in 1949 showed that for plants deprived of nitrogen, and also for those in which flowering was greatly accelerated, the spike at double-ridge formation was considerably shorter than 1·6 mm.: it was, therefore, necessary to determine for each variant the mean spike length of those apices which had just reached the stage of double ridges, and from these data to calculate proportionally a new scale relating spike length and progress towards differentiation.

The advantage of a scoring method is that it enables a number indicative of progress to flowering to be assigned to plants which have not reached anthesis at the time of sampling; plants which have already flowered can be assigned 'scores' on the same scale by adding 49 (the 'score' which indicates that anthesis is attained on the day of sampling) to the number of days that have elapsed since flowering. In this way an average measure of flowering behaviour may be obtained from a sample which includes both immature plants and those which have already flowered. Other 'scoring' methods have been suggested by Feekes (1941) (quoted by van de Sande Bakhuyzen, 1947), Hänsel (1953), and others. Alternative methods of assessing the flowering behaviour of a group of plants, e.g. 50 per cent. point, usually indicate only the number of plants past a particular stage and as no account is taken of the variable individual progress, such data do not lend themselves to statistical analysis. The linearity of progress in 'score' makes it a more useful datum than the length of the spike, since the latter increases exponentially, and therefore arithmetic means are not appropriate. In periodical dissections a record of *mean scores* fails to indicate at what time differentiation begins; for this reason the percentage of plants at or past differentiation (double ridges) was also recorded. This exposes the great variability of the response of the plants, for several weeks may elapse between the appearance of double ridges and the time at which all the plants in a sample have passed this stage.

EXPERIMENTAL RESULTS

a. Flowering in relation to level of nitrogen supply

For most of the experiments described the amount of nitrate added to the sand culture was reduced to 1/10th that employed in previous work; in this way the abundant tillering of the unvernalised plants was avoided, and manipulation facilitated. It was, however, necessary to examine in more detail the effect of nitrogen nutrition on spike development and flowering behaviour. In 1948 three levels of nitrogen were used, viz. 1/10, 1/50, and nil respectively, applied to winter rye vernalised for various periods between 0 and 8 weeks. In 1949 two levels, standard and 1/10, were used, and the plants grown in continuous light. The results are entered in Table 1 (*a*, *b*, and *c*).

Progress to flowering, as determined either by score or by leaf number, is unaffected by nitrogen level in highly vernalised plants: in unvernalised and slightly vernalised plants, however, nitrogen deficiency *retards* progress to flowering. The data in Table I*b* show the expected differences in ear length and spikelet number due to variation in nitrogen supply, but the effect on time of flowering is negligible. The early work of Purvis (1934) is thus confirmed. Borodin (1931), on the other hand, found in spring barley that the retarding effect of short days could be overcome by withholding nitrogen; while von Denffer (1950) showed that low nitrogen accelerates flowering and reduces leaf number in spring barley grown in short as well as in long

TABLE I*a*

The Effect of Nitrogen Supply on Flowering of Winter Rye vernalised for Different Periods (No. of Replicates in brackets). Plants grown in Natural Summer Days. 1948. Sowing date, April 26

N level	0		$\frac{1}{50}$ standard		$\frac{1}{10}$ standard	
V in weeks	'Score'.	Leaf No.	'Score'.	Leaf No.	'Score'.	Leaf No.
0	17 (8)	18·5	32 (6)	19·6 (5)	31 (8)	17·9
3	78 (12)	13·5	72 (5)	15·8	77 (11)	14·1
4	93 (16)	11·5	89 (14)	11·6	90 (17)	11·8
5	99 (20)	10·0	100 (13)	9·9	99 (16)	10·3
6	106 (16)	9·4	106 (19)	9·8	107 (16)	10·1
8	113 (14)	8·7	113 (19)	8·0	114 (18)	7·8

TABLE I*b*

Effect on Flowering of reducing N to $\frac{1}{10}$ Standard. Plants grown in Continuous Light. 1949. Sowing date, May 1

N level.	Full standard.				$\frac{1}{10}$ standard.			
V in weeks.	'Score'.	Leaf No.	Ridge No.	Ear length. mm.	'Score'.	Leaf No.	Ridge No.	Ear length. mm.
0	80	15·0	34	78	75	14·3	26	61
3	110	11·6*	30	72	113	9·7*	19	48
5	124	8·6*	21	59	124	7·6*	15	41
9	128	6·2	13	43	129	6·4	11	32
13	129	5·9	13	42	128	5·9	10	30

* Highly significant difference (P = 0·01) between standard and 1/10 standard nitrogen level.

TABLE I*c*

Effect of Low Nitrogen on Flowering in Short Days

N level.	None.			$\frac{1}{10}$ standard.		
V in weeks.	'Score'.	Leaf No.	% past 'double ridges'.	'Score'.	Leaf No.	% past 'double ridges'.
4	20*	19·3	50	31	20·5	100
6	25*	18·0	73	30	18·8	83
8	32	16·8	100	36	17·6	100

* As these means include undifferentiated plants, and are therefore based on spike lengths, the score is depressed by low nitrogen.

days. This is not the case in 'Petkus' winter rye, as the data in Table I*c* show. Under short-day conditions again, flowering is somewhat delayed by the absence of added nitrogen. The reduction in score may in some measure be due to the shortcomings of the scoring method; the relation of spike length to developmental progress noted above differs with low nitrogen supply and the scores assigned to the nitrogen-deficient plants are probably too low. There is thus no evidence for any hastening of flowering in rye by reduction of nitrogen supply, but on the contrary there may be a retardation: certainly increased nitrogen supply does not delay flowering in winter rye. It is therefore safe to make comparisons between sets of plants even though the nitrogen level differs, and the work reported in this paper may legitimately be compared with previous published work.

b. Flowering of spring and winter rye under different daylengths and degrees of vernalisation

The flowering behaviour of plants under continuous light, normal daylength, and short days of 10 hours' duration was compared, the plants having been previously vernalised for various periods. Mean scores and leaf numbers at 19 weeks from planting are shown for different years in Table II*a*, *b*, and *c*.

Continuous light. The accelerating effect of continuous light as compared with normal summer days is clearly shown for spring rye and for winter rye at all degrees of vernalisation. This effect is greatest in the unvernalised series and diminishes as vernalisation is prolonged; thus in 1948 the difference in score of 54·4 between unvernalised plants in continuous light and in long days is reduced by 3 weeks' vernalisation to 14·0 and thereafter remains nearly constant. Similar relations are seen in data for 1949 and 1950 (Table II*b* and *c*). Thus in 1950 a maximal score difference of 43 occurs with 2 weeks' vernalisation and after 4 weeks' vernalisation the difference again falls to 13, and remains nearly constant up to full vernalisation. Despite the evidence of a seasonal variation, the general interaction of daylength and duration of vernalisation appears to hold.

Short days. In 10-hour days the effect of vernalisation on progress to flowering is largely suppressed. The score corresponding with initiation of flowering, namely 21, is, however, attained at all levels of vernalisation, and (except for 1950) also in the unvernalised controls. In 1950 the mean score for unvernalised plants was 17·4, but 37 per cent. of the replicates had passed the stage of initiation of flowering. Comparing the scores in short and normal days it is seen that differentiation in unvernalised plants is greater in long days, which is not in agreement with the previous finding (Purvis, 1934) that short days are more favourable to initiation in unvernalised plants. This discrepancy will be considered again later.

c. Progress towards flowering in relation to daylength

The data so far considered show the stage of differentiation reached after 19 weeks' growth. In order to ascertain the time at which flower initiation was reached, samples were taken at intervals in 1949 and 1950 and the apex of the main shoot dissected.

The data in Table III refer to experiments of (*a*) 1949 and (*b*) 1950. The scores reached after varying numbers of weeks from planting out are pre-

TABLE II

Effect of Continuous Light, Normal Daylength, and Short Days on Flowering of Vernalised and Unvernalised Winter Rye and of Spring Rye. Mean Scores and Leaf Numbers 19 Weeks after Planting

a. 1948. Planted April 26–29 under supplementary light of 160–750 lux. Number of replicates approximately 20

Weeks vernalised.	24 hours.		Normal long days.		10 hours.	
	Score.	Leaf No.	Score.	Leaf No.	Score.	Leaf No.
0	85·5	13·7	31·1	17·9	—	—
3	91·6	13·1	77·1	14·1	—	—
4	104·8	10·5	89·8	11·8	30·9	20·5
5	114·1	9·0	99·2	10·3	—	—
6	123·0	7·6	106·6	10·1	30·2	18·8
8	124·7	6·7	114·1	7·8	36·2	17·6

b. 1949. Planted May 1–5, under supplementary light of 160–210 lux. Number of replicates approximately 20

Weeks vernalised.	24 hours.		Normal long days.		10 hours.	
	Score.	Leaf No.	Score.	Leaf No.	Score.	Leaf No.
0	75·0	14·3	28·6	—	24·1	18·4
1	87·4	13·2	—	—	25·0	18·3
2	96·6	12·1	62·5	15·0	26·5	18·0
3	112·8	9·7	—	—	—	—
4	119·9	8·9	114·1	10·6	—	—
5	123·9	7·6	—	—	—	—
6	126·4	7·5	123·1	9·2	—	—
7	127·4	6·8	—	—	—	—
9	128·7	6·4	122·4	7·2	34·4	15·7
11	128·9	6·0	—	—	33·0	14·4
13	127·9	5·9	—	—	32·4	15·2
Spring rye	129·3	6·5	—	—	33·6	14·8

c. 1950. Planted May 1–5, under supplementary light of 160–210 lux. Number of replicates approximately 35

Weeks vernalised	24 hours.		Normal long days.		10 hours.	
	Score.	Leaf No.	Score.	Leaf No.	Score.	Leaf No.
0	56·1	17·2	21·6	22·1	17·4	21·8
1	58·9	16·9	20·8	21·2	—	—
2	81·9	14·0	38·9	19·1	—	—
3	103·7	11·5	71·6	15·5	—	—
4	115·9	—	102·5	—	—	—
11	127·3	6·2	119·6	7·3	34·8	—
Spring rye	126·3	6·6	118·7	7·6	32·9	16·0

sented, together with the percentage of replicates attaining flower initiation (figures in brackets). The following conclusions may be drawn:

1. In spring and fully vernalised winter rye flower initiation is complete or almost so 3 weeks after planting out in continuous light. In normal

days full initiation is delayed by approximately 1 week, but has already begun in 3 weeks. Short-day conditions greatly delay initiation, which begins after 6 weeks (1949) or 7 weeks (1950) respectively.

TABLE III

Progress towards Flowering of Highly Vernalised and Unvernalised Winter Rye, and of Spring Rye, under Conditions of Continuous Light, Normal Long Days, and Short Days. 'Scores' are based on approximately 12 replicates (36 at 19 Weeks). In brackets, percentage of Replicates attaining Flower Initiation

a. Experiment of 1949. Winter rye, planted May 1–5

Weeks from planting.	9 weeks vernalised		Unvernalised winter rye.	
	24-hr. day.	10-hr. day.	24-hr. day.	10-hr. day.
3	30·6 (100)	—	4·9 (0)	—
4	33·5 (100)	9·5 (0)	7·0 (0)	6·2 (0)
5	35·6 (100)	—	6·5 (0)	—
6	43·3 (100)	14·9 (17)	6·1 (0)	8·8 (0)
8	—	—	12·0 (25)	—
9	—	20·6 (53)	—	9·9 (0)
10	—	—	24·4 (88)	—
12	—	30·3 (100)	—	18·6 (33)
15	—	30·3 (92)	—	13·9 (14)
19	128·7 (100)	34·4 (100)	75·0 (100)	24·1 (67)

b. Experiment of 1950. Winter and spring rye, planted May 1–5

Weeks from planting.	Spring rye			Unvernalised winter rye.		
	24-hr. day	Normal day.	10-hr. day.	24-hr. day.	Normal day.	10-hr. day.
1	7·2 (0)	6·3 (0)	—	4·1 (0)	4·3 (0)	4·2 (0)
2	11·7 (0)	7·2 (0)	5·1 (0)	4·8 (0)	4·8 (0)	5·2 (0)
3	24·7 (90)	14·8 (17)	—	5·7 (0)	5·6 (0)	5·4 (0)
4	—	25·7 (100)	7·2 (0)	6·6 (0)	6·3 (0)	6·1 (0)
6	—	—	—	7·7 (0)	7·1 (0)	7·4 (0)
7	—	—	13·1 (8)	—	—	—
8	—	—	—	11·0 (8)	8·1 (0)	7·3 (0)
10	—	—	21·6 (75)	13·9 (0)	10·7 (0)	10·2 (0)
12	—	—	—	24·8 (75)	9·5 (0)	12·4 (18)
14	—	—	30·1 (100)	—	—	—
15	—	—	—	—	17·1 (33)	14·2 (8)
19	126·0 (100)	119·0 (100)	32·9 (100)	56·1 (100)	21·6 (47)	17·4 (37)

2. In unvernalised winter rye initiation of floral primordia is delayed in all daylengths, occurring after 8 weeks in continuous light, 12 weeks in 10-hour days, and not until 15 weeks in normal summer days. In the short-day series variability among replicates is very large so that the percentage of initiated plants increases very slowly and by 19 weeks the normal-day plants have overtaken the short-day set. This more rapid progress in flowering is seen in the rate of increase of the scores after initiation has begun. In continuous light even unvernalised

plants have reached the stage of anthesis (score 49) in 19 weeks, while in normal days stem extension has not yet begun.

(*d*) *Progress of differentiation in the apex in relation to daylength*

In the course of examination of the apices of the main shoots in samples taken at intervals, details of the stage of differentiation were also recorded as previously stated (p. 83). By subtracting the final leaf number from the total number of primordia, the number of prospective floral primordia present at any time during development was determined. The relevant data obtained from the 1950 experiment are entered in Tables IV and V for spring rye and unvernalised winter rye respectively, when grown in continuous light, normal days, or 10-hour days. The age of the plant at which first floral primordia (double ridges) were noted is indicated by heavy type.

TABLE IV

Progress of Leaf and Primordium Formation in Spring Rye under Continuous Light, Normal Long Days, and Short Days (10 hours). Replication approximately 12. Mean Number of Lateral Appendages. Experiment of 1950.

	Day-length.	Weeks from planting.							
		1	2	3	4	7	10	14	19
Emerged leaves	C.L.	1·0	1·8	3·0	—	—	—	—	6·6
	L.D.	1·0	1·4	2·7	4·2	—	—	—	7·6
	S.D.	—	1·7	—	3·5	6·8	9·8	12·7	14·5
Total determinate leaf primordia	C.L.	4·5	5·6	6·9	—	—	—	—	6·6
	L.D.	4·4	5·2	7·3	7·8	—	—	—	7·6
	S.D.	—	5·3	—	8·8	12·9	15·7	16·2	16·0
Indeterminate and floral primordia	C.L.	1·0	2·0	**11·5**	—	—	—	—	11·1
	L.D.	1·0	1·2	**4·8**	17·7	—	—	—	15·7
	S.D.	—	1·5	—	3·4	**14·9**	27·7	37·8	34·2
Total primordia	C.L.	5·5	7·6	18·4	—	—	—	—	18·7
	L.D.	5·4	6·4	12·1	25·5	—	—	—	23·3
	S.D.	—	6·8	—	12·2	27·8	43·4	54·0	50·2

Heavy type indicates first appearance of floral primordia.

Spring rye. In continuous light double ridges were observed after 3 weeks. At this time on the average 18·4 primordia had been formed by the apex; of these 6·9 were determinate leaves so that at most 11·5 were available to form spikelets. Since at 19 weeks only 11·1 spikelets were present, it is clear that by 3 weeks the ear was already completely determined, and no further spikelet primordia were formed. At this stage only three emerged leaves were present. Since the final leaf number in this series was 6·6, whereas at 2 weeks 7·6 primordia had been formed, it is clear that one primordium of these would eventually form a spikelet. Initiation had thus already taken place at 2 weeks from planting out at a time when no morphological sign of flowering had yet appeared. Initiation thus antedated double-ridge formation by 1 week. At the time of initiation only two leaves had emerged, of which only one was fully developed.

In normal long days initiation and double-ridge formation both occurred at 3 weeks, while in short days both these stages were reached in 7 weeks. In normal long days the ear was fully determined by 4 weeks, i.e. 1 week after initiation, while in short days spikelet formation continued for a further 7 weeks and the ear was not fully determined until 14 weeks from planting out. The number of spikelets (each bearing two flowers) in the ear were 11·1, 15·7, and 34·2 respectively in C.L., L.D., and S.D.

TABLE V

Progress of Leaf and Primordium Formation in Unvernalised Winter Rye under Continuous Light, Normal Long Days, and Short Days (10 hours). Replication approximately 12. Mean Number of Lateral Appendages. Experiment of 1950.

	Day length.	Weeks from planting.									
		1	2	3	4	6	8	10	12	15	19
Emerged leaves	C.L.	0·8	1·1	2·7	4·0	6·0	7·8	10·2	12·4	—	17·2
	L.D.	1·0	1·0	3·0	3·9	5·7	7·7	10·0	11·1	14·5	17·1
	S.D.	1·0	1·3	2·6	3·9	5·6	7·3	8·5	10·0	13·3	16·5
Total determined leaf primordia	C.L.	4·0	4·8	7·1	8·6	12·2	14·0	15·9	16·3	—	17·2
	L.D.	4·1	4·8	7·3	8·3	10·9	13·2	16·7	16·4	19·8	22·1
	S.D.	4·2	5·0	7·1	8·3	10·3	13·1	14·8	15·4	17·8	21·8
Indeterminate and floral primordia	C.L.	0·8	1·0	1·4	2·5	4·3	**8·3**	14·0	25·0	—	25·9
	L.D.	1·0	0·7	1·1	1·9	2·5	5·3	9·6	8·4	**18·7**	24·4
	S.D.	1·0	1·0	1·2	1·9	3·9	4·8	9·2	**10·8**	13·9	19·5
Total primordia	C.L.	4·8	5·8	8·5	11·1	16·5	22·3	29·9	41·3	—	43·1
	L.D.	5·1	5·5	8·4	10·2	13·4	18·5	26·3	24·8	38·5	46·5
	S.D.	5·2	6·0	8·3	10·2	14·2	17·9	24·0	26·2	31·7	41·3

Heavy type indicates first appearance of floral primordia.

It is clear that the rate of primordium formation in the apex increases rapidly after initiation, and that this rapid differentiation begins before any morphological sign of flower differentiation appears, again indicating that initiation appreciably precedes double-ridge formation.

Unvernalised winter rye. The behaviour of unvernalised winter rye differs markedly from that of spring rye. Until 3 weeks from planting the rate of primordium formation in the apex is independent of daylength, at which time 7 leaf primordia have been laid down. After this time the differentiation rate in C.L. begins to overtake that in L.D. and S.D. and at 8 weeks double ridges first appear. Unlike spring rye, however, in continuous light, unvernalised winter rye continues to produce spikelet initials for another 4 weeks. In both long and short days, primordium formation continues at an almost constant rate until double ridges first appear at 12 weeks in S.D. and at 15 weeks in L.D. The time of first initiation is thus actually earlier in S.D. than in L.D. Primordium formation continues in both daylengths and the final number of spikelets (floral pairs) in the ear is 25·9, 24·4, and 19·5 in C.L., L.D., and S.D. respectively. Ear size in unvernalised winter rye is much less affected by daylength than in spring rye, and the relation with daylength is in the opposite sense.

TABLE VI

Effect of Vernalisation on Growth of Apex. Mean Apex Length (mm.). Experiment of 1950. Number of Replicates approximately 12

(*a*) Under continuous light

	Weeks vernalised.					
Weeks from planting.	0 control.	1	2	3	11	Spring rye.
1	0·072	0·119	0·129	0·118	0·178	0·117
2	0·120	0·109	—	—	0·330	0·245
3	0·174	0·20	0·21	0·19	**1·27**	**0·91**
4	0·23	0·24	0·23	0·27	—	—
5	0·31	0·29	0·29	**1·20**	—	—
8	**0·58**	**0·50**	**7·7**	11·2	—	—
10	0·71	4·4	15·9	34·4	—	—
12	8·3	—	—	—	—	—
19	55·9	59·2	51·6	52·4	33·0	33·9

Heavy type indicates first appearance of floral primordia (double ridges).

(*b*) Under normal long days

	Weeks vernalised.					
Weeks from planting.	0 control.	1	2	3	11	Spring rye.
1	0·081	0·119	0·133	0·101	0·185	0·106
2	0·119	0·118	—	—	0·32	0·135
3	0·168	0·167	0·155	0·179	**0·60**	**0·39**
4	0·210	0·233	0·238	0·206	1·97	1·29
6	0·27	0·217	0·276	**0·38**	—	—
8	0·34	0·30	0·26	4·11	—	—
10	0·51	0·36	0·46	12·7	—	—
12	0·52	**0·81**	**5·07**	30·0	—	—
15	**0·88**	11·0	20·9	38·0	—	—
19	2·49	7·9	30·6	44·1	44·8	44·0

Heavy type indicates first appearance of floral primordia (double ridges).

(*c*) Under short (10-hour) days

Weeks from planting.	Unvernalised winter rye control.	Spring rye.
1	0·079	—
2	0·14	0·14
3	0·15	—
4	0·21	0·28
6	0·29	—
7	—	**0·64**
8	0·28	—
10	0·47	1·76
12	**0·58**	—
14	—	3·55
15	0·70	—
19	1·1	13·6

Heavy type indicates first appearance of floral primordia (double ridges).

It may be pointed out that the rate of emergence of leaves in both spring and winter rye is independent of daylength, and approximates one leaf per week.

(*e*) *The growth rate of the apex in relation to daylength and duration of vernalisation*

At the time of dissection of the samples taken in 1950 during growth of the plants the length of the apex was measured from the extreme tip to the lowest primordium just overlapped by the developing leaf inserted immediately below.

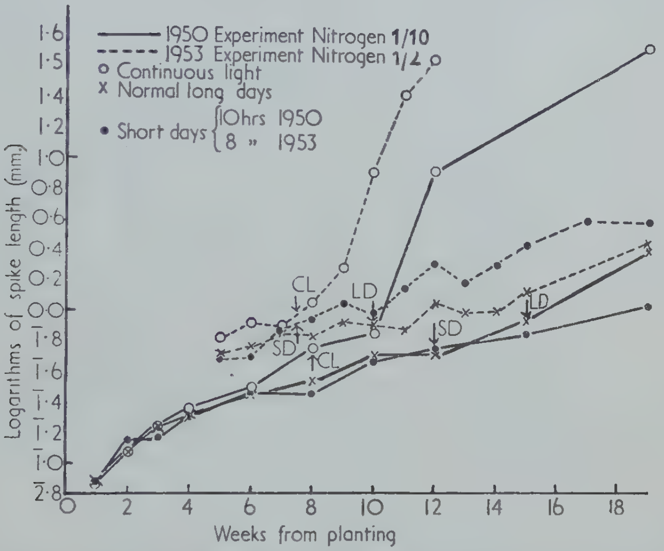

FIG. 3. Relative growth rate of apex of main shoot of unvernalised winter rye grown in three daylengths and at two nitrogen levels. The arrows denote the times of appearance of 'double ridges' in the various daylengths.

These data, for varying periods of vernalisation, under conditions of (*a*) continuous light, (*b*) normal days, and (*c*) short days are entered in Table VI. Confining attention first to the behaviour of the unvernalised winter rye controls, the results are presented graphically in Fig. 3 (solid lines) in which the logarithms of the mean spike lengths are plotted against weeks from planting. The slope of the curves shows the relative growth rate under the different daylengths. By 8 weeks the relative growth rate of the plants in C.L. has increased, at which time double ridges have appeared. Initiation in C.L. is followed by a very rapid increase in the relative growth rate, and the grand period of growth of the ear begins.

The plants grown in L.D. show a constant relative growth rate up to 12 weeks, followed by an increase and double-ridge formation at 15 weeks. The grand period of growth of the ear thus begins around 12 weeks, but the rate of growth of the ear is slow compared with that in C.L.

TABLE VII

Progress of Spike Development in Unvernalised Winter Rye, in Continuous Light, Long Days, and Short Days, using Nitrogen at Half Standard Level. Experiment of 1953. Replication 10

Daylength (hrs.).		Weeks from planting.												
		5	6	7	7½	8	9	10	11	12	14	15	17	19
Scores	24 (C.L.)	10·1	12·1	11·7	**16·0**	16·0	23·1	27·4	31·9	35·6	40·4	46·5	62·7	84·4
	17 (L.D.)	8·7	9·5	10·8	10·7	10·6	12·7	**12·2**	11·8	17·4	17·2	19·3	—	24·4
	8 (S.D.)	8·2	8·8	10·6	**15·2**	12·3	19·3	15·6	20·2	23·1	24·4	24·7	—	31·5
Spike length (mm.)	24 (C.L.)	0·65	0·83	0·80	**1·32**	1·15	1·97	8·1	25·8	44·5	—	—	—	—
	17 (L.D.)	0·54	0·59	0·71	0·70	0·69	0·84	**0·79**	0·74	1·15	0·98	1·35	—	2·80
	8 (S.D.)	0·48	0·50	0·78	**0·93**	0·88	1·11	0·95	1·40	2·05	2·00	2·67	3·88	3·88

Heavy type indicates first appearance of flower primordia.

N.B. In this experiment the natural summer day was increased to 17 hours by supplementary artificial light (160–210 lux). Short days were of 8 hours' duration only.

In S.D. the relative growth rate remains constant up to 19 weeks. In short days, therefore, there is no grand period of growth of the ear, which continues to enlarge at a constant relative rate. In one important respect the results of 1950 differ from the previous observation (Purvis, 1934) on the growth of unvernalised plants, namely, that the relative growth rate is similar in short days and in long days and finally higher in long days, whereas it was previously claimed that in short days the relative growth rate is greater than in long days. There is, however, the difference that whereas in 1932 and 1933 full nitrogen was supplied, in 1950 the level was reduced to 1/10th the full supply, and it appeared possible that the low level of nitrogen may have reduced apical growth without affecting flowering behaviour. To test this hypothesis the experiment was repeated in 1952 and 1953, using a higher nitrogen level, viz. half the standard application. The results obtained in 1953 are shown in Table VII and graphically in Fig. 3 (broken line).

It will be noted that in 1953 the short daylength used was 8 hours in place of the 10-hour day used in 1950, and normal summer days were extended to a constant 17-hour duration by weak artificial light (160–210 lux). In 1952 another experiment had been carried out with unvernalised winter rye in which days of 17, 10, and 8 hours were compared. The spike lengths at various times after planting are shown in Table VIII below.

TABLE VIII

Progress of Spike Development in Unvernalised Winter Rye in Long and Short Days, using Nitrogen at Half Standard Level. Experiment of 1952. Replication 10

Daylength in hours.		Weeks from planting.							Final ridge
		4	6	8	10	12	15	19	No.
Score	17	7·1	9·4	11·8	13·8	14·4	14·8	25·7	3·9
	10	6·0	8·4	11·4	15·6	17·0	25·0	28·2	3·1
	8	6·2	9·4	15·0	19·5	14·5	23·5	24·7	2·9
Spike length (mm.)	17	0·37	0·58	0·80	**0·86**	0·97	0·97	2·20	—
	10	0·25	0·49	**0·61**	0·83	1·26	2·00	2·50	—
	8	0·24	**0·54**	0·89	1·13	0·93	2·09	2·35	—

Heavy type indicates the first appearance of flower primordia.

In this experiment the natural summer day was increased to 17 hours by means of supplementary artificial light (160–210 lux).

In both experiments the spike length was greater in short days than in long days, particularly in 1953 when the values were consistently higher. Double ridges in 1952 appeared after 6 weeks in 8-hour days, 8 weeks in 10-hour days, and at 10 weeks in 17-hour days. In 1953 the times to the appearance of floral primordia were 7½ weeks in short days and 10 weeks in long days.

In the experiment of 1953 the continuous light and short-day series both received daily 8 hours of full daylight, at the end of which both sets were removed to a dark shed in which the short-day series was kept in darkness and the continuous light series received weak artificial light. Both series were thus kept at approximately the same temperature and both received the same measure of full daylight. The long-day series could not receive comparable treatment: these plants remained in the open in 17-hour days consisting of full daylight and the necessary supplement of weak artificial light. The nightly minimum temperatures were about 5° C. lower for the long-day plants than for those in darkness in the shed, and 6° C. lower than for the continuous light set which had supplementary light in the shed. There was, thus, at night a very slightly higher temperature in favour of the continuous light set over the short day and a considerable difference between short-day and long-day plants in favour of the former series.

Comparing the growth rates in 1950 with those in 1953, as shown in Fig. 3, it appears that the spike lengths were higher in 1953 than in 1950, but the relative growth rates were similar as seen from the slopes of the appropriate curves. The plants in short days were slightly behind those in long days until 7 weeks after planting, but subsequently the short-day plants overtook the long-day series and the lead was maintained. The point at which the curves cross corresponds with the formation of those simple ridges which later would give rise to spikelets; visible doubling of the ridges followed a week later. The fact that for the first 6 weeks spike growth was slower than in long days indicates that the increased rate of elongation after 7 weeks in the short-day series was not due to the higher temperature during the dark period in the shed. The time at which spike length in short days overtakes that in long days marks the limit of duration of short-day treatment resulting in optimal short-day induction. Although the experiment was not designed to investigate short-day induction, a limited number of plants were transferred from short days to long days treatment 7 weeks from planting. The final score of the transferred plants after 19 weeks was 42·7 compared with 24·4 for plants in long days throughout; short-day induction was therefore considerable.

The important conclusions emerging from these experiments are: (1) In unvernalised winter rye continuous light has a direct effect on the initiation of flowering as well as on the grand period of growth of the ear and the extension growth of the stem. (2) With adequate nitrogen short days also hasten initiation and increase the relative growth rate of the apex above that of plants in normal long days; hence two distinct ranges of daylength are optimal for initiation and development of the spike.

f. Interaction of vernalisation and daylength on growth of the apex

To return to the data presented in Table VI, the interaction of daylength and degree of vernalisation on growth of the apex and initiation of flowering remains to be considered.

The data of apex length are presented in Fig. 4 as logarithms of spike length plotted against weeks from planting. The two sets of curves, *a* and *b*,

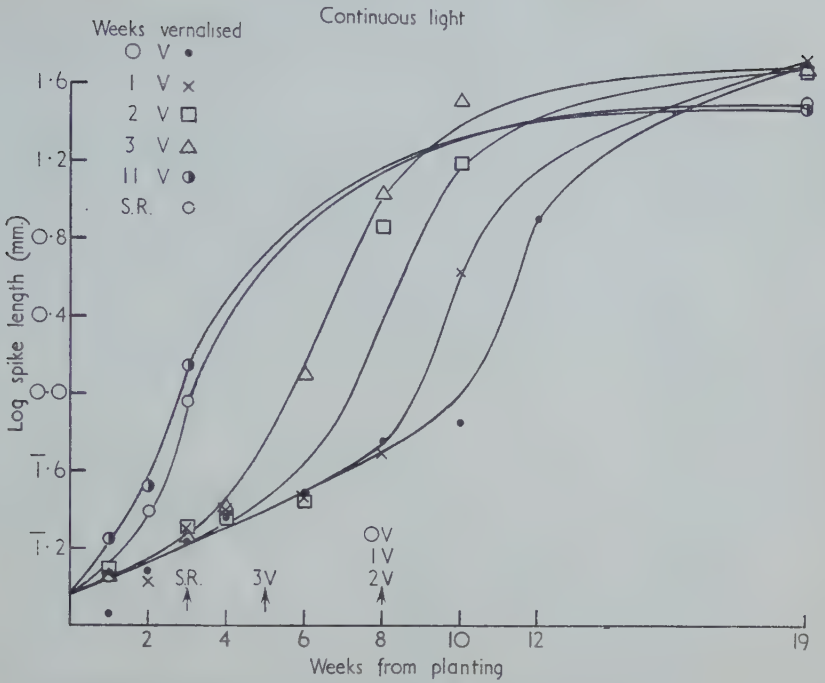

FIG. 4*a*. Relative growth rate of apex of main shoot of plants with various durations of vernalisation grown under continuous light.

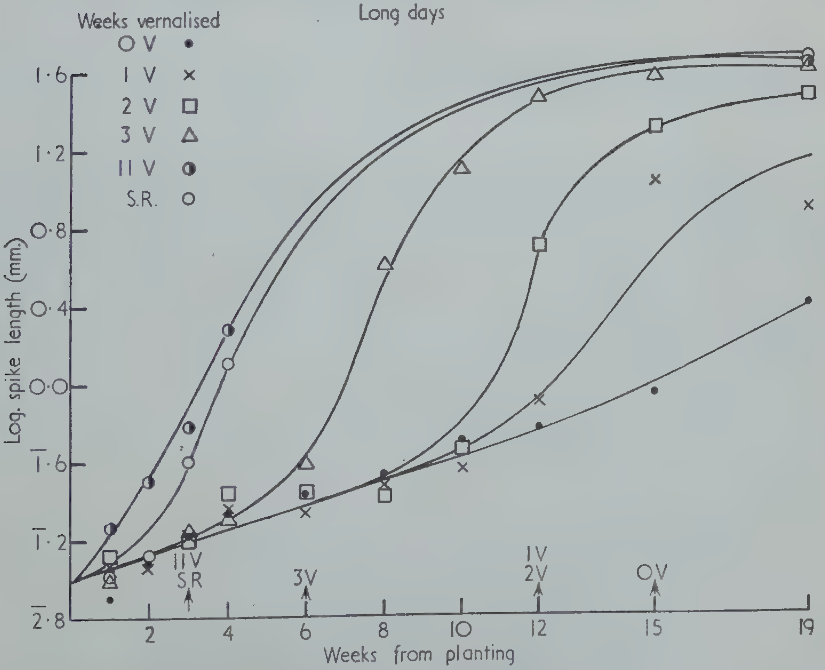

FIG. 4*b*. As Fig. 4*a*, but plants grown in normal daylength. The arrows denote time of appearance of 'double ridges'.

refer to the behaviour in continuous light and natural long days respectively. Arrows indicate time of appearance of double ridges in the various vernalised series. In both daylengths the relative growth rate of the undifferentiated spike remains constant; in continuous light up to the 10th week and in long days up to the 15th week. The relative growth rate is, however, higher in continuous light than in long days, indicating that daylength affects the apical growth even before initiation is manifest.

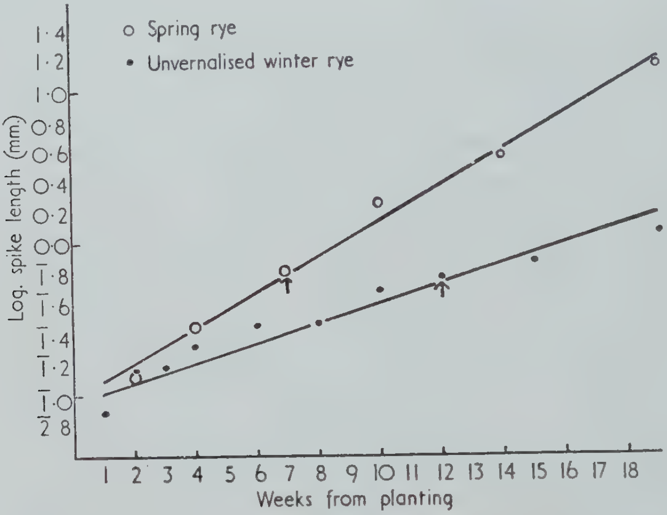

FIG. 5. Relative growth rate of apices of main shoot of unvernalised winter rye and of spring rye grown in short days. The arrows denote time of appearance of 'double ridges'.

As the period of vernalisation is increased the time to double-ridge formation is progressively reduced, and immediately on initiation the relative growth rate increases abruptly. The fully vernalised winter rye given 11 weeks' vernalisation shows in both daylengths a slightly earlier initiation and rise in the relative growth rate of the apex than does spring rye. The effect of only 1 week of vernalisation is clearly shown in both daylengths. The effect of continuous light is again shown to be twofold at all degrees of vernalisation: first, an increase in the relative growth rate before initiation; second, an earlier initiation of flowering than in long days, and a marked differential increase of the growth rate of the developing ear, greatest in the unvernalised plants, and becoming less as the period of vernalisation is increased.

A comparison of apical growth in spring rye and in unvernalised winter rye in *short days* is shown in Fig. 5. The relative growth rate of unvernalised winter rye is less than that of spring rye; it is, moreover, similar to that in normal long days, but as stated earlier the grand period of growth is absent in short days. In general it appears that after initiation has occurred the onset of the grand period of spike growth is dependent on the daylength;

it is very marked in continuous light, little in long days, and absent in short days.

g. The reversal of initiation

The question arises whether initiation once achieved is stable, or, in part, reversible when conditions inhibiting to flowering are later imposed. Various structural abnormalities in ear structure are commonly found in cereals, especially in autumn, when flowering is retarded by short days. Such abnormalities have been described and figured by Forster, Tinker, Vasey, and Wadham (1932), McKinney and Sando (1934), and by Sharman (1944). Wycherley (1952) found that in *Cynosurus cristatus*, plants exposed to long days followed by short days produced inflorescences in which spikelets were vegetatively proliferated. These, when detached, were capable of rooting and growing. If long days again followed short days, the vegetative bud still attached to the mother plant formed inflorescences, those growing detached remaining vegetative.

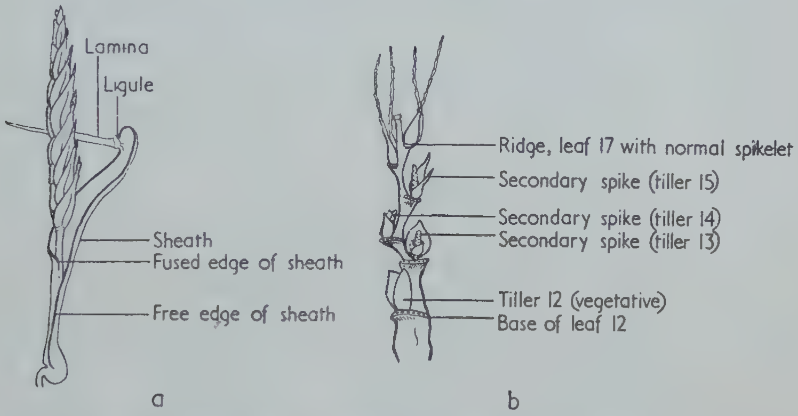
Lamina
Ligule
Sheath
Fused edge of sheath
Free edge of sheath
a
Ridge, leaf 17 with normal spikelet
Secondary spike (tiller 15)
Secondary spike (tiller 14)
Secondary spike (tiller 13)
Tiller 12 (vegetative)
Base of leaf 12
b

FIG. 6. Diagram of abnormal ear development induced by transfer to short days of unvernalised winter rye plants originally in long days. For details see text.

In the cereals, however, regeneration of complete plants from spikelets in the ear has not, to the writers' knowledge, been observed, and the abnormalities are limited to a very few nodes at the base of the ear. Upper spikelets without exception produced normal flowers.

The following types of abnormality have been encountered in the present work. (1) Leaves are formed at the lower ear internodes subtending spikelets. These leaves are small; they may be green or may be white and membranous. (2) The spikelets may continue their apical growth thus forming secondary ears similar to those found by Wycherley in *C. cristatus*. These may have two flowers at the base, or two or more leaves. This anomaly may be combined with the formation of supplementary leaves at the base of the ear as described under (1). In one specimen tertiary ears were observed. (3) One or two internodes of the ear may undergo extension growth. (4) The insertion

TABLE IX

Transfer of Spring Rye from Natural Long Days (L.D.) to 10-hour Days (S.D.). Scores at 77 days

Days in L.D.	Days in S.D.	Final score.	Leaf No.		Notes on structure								Replicate.
			Normal	Supplementary.	Plants 1	2	3	4	5	6	7	8	
7	70	37±0·7	13·7±0·4	0·8	F	DEF	CDEF	CEF	AF	E	E	—	7
14	63	41±2·3	7·8±0·3	2·3	D	CF	DF	DEF	DE	DEH	D	DF	8
21	56	68±1·1	7·9±0·1	—	Normal. Poor growth								12
28	49	75±3·8	7·0±0·0	—	Normal growth controls								7
77	0 (control)	77±0·5	6·8±0·2	—	Normal								10

Key: A = Normal.
B = Some spikelets with continuous apical growth, bearing several flowers.
C = Lowest spikelet subtended by green leaf.
D = Lowest spikelet subtended by white membrane.
E = Spikelets replaced by spikes.
F = Many sterile spikelets.
G = Anomalous attachment of leaf.
H = Main axis of ear branched.

of supplementary leaves is often anomalous, one margin of the leaf sheath being fused with the stem over a length of up to 1 cm. and the other margin free. Some of these anomalies are shown in Fig. 6.

In 1936 the after effects of both long and short days on spring rye were investigated. As previously noted, spring rye is a typical long-day plant. The results are shown in Table IX.

When long days preceded short days so that some degree of initiation occurred before transfer to short days, it was found that 4 weeks in long days sufficed to achieve a maximal score, equal to that in continued long days. After 3 weeks in long days subsequent short days delayed flowering by only 10 days. Both these treatments, however, checked vegetative growth. With only 2 weeks in long days there was considerable delay in flowering accompanied by increased leaf number, and, moreover, structural abnormalities appeared, similar to those listed above. Furthermore, these abnormalities did not appear when the period in the non-inductive daylength (10 hours) preceded that in long days.

TABLE X

After Effect of Short Days (10 hours) on Spring Rye transferred to Normal Long Days

Preliminary short days.	Subsequent long days.	Total days to anthesis.	Leaf number.	Replicates.
0	50	50±0·5	6·8±0·2	10
33	39	72±0·6	12·3±0·2	7
42	38	80±2·3	14·0±0·4	4
49	38	87±0·3	15·0±0·3	5
56	40	96±1·0	15·0±0·3	5
113	0	Failed to flower	18·0±0·6	6

Table X gives the results of such transfer. The same number of long days was then required for flowering whatever the duration of the short-day exposure; this period was less than the number of long days required without any preliminary exposure to short days. These treatments gave rise to entirely normal ears.

h. Minimal leaf number

As a result of earlier work (Purvis, 1934; Purvis and Gregory, 1937) it was suggested that in rye a 'minimal leaf number' exists; that is, that before flower initiation can begin, a minimal number of plastochrones must elapse. From the early results it was thought the number of leaves characteristic of the termination of the vegetative phase was seven, and the differentiation of this complement of leaves marked 'ripeness to flower'. Until requisite continuous light facilities were available at the end of the war no experiments could be carried out to ascertain whether continuous light could reduce the leaf number to a lower level before initiation. As the ripe grain already contains 3 to 4 primordia a minimal leaf number of 7 would mean that at least

3 new primordia must be laid down before flowering can begin: on this basis the concept of a minimal leaf number was framed.

The accelerating effect of continuous light was accompanied by a reduction in leaf number to 5·9 (Table II*b*, 13 weeks' vernalisation in continuous light). The question thus arises whether in fact the plant is 'ripe to flower' immediately after germination as soon as the first green leaf emerges. Two modes of approach to this problem were explored. (1) The grain was kept continually in light from the time of soaking and throughout the period of 8 weeks' vernalisation. (2) By the use of prematurely harvested grain, the embryos of which may have as few as two primordia (Nutman, 1941); such grain can be successfully vernalised (Hatcher and Purvis, 1945).

TABLE XI

Mean Grain Weight of Prematurely Harvested Grain and of Mature Spring Rye

Maturity of seed (days from anthesis at harvest).	Nitrogen supplied to parent plant.	Weight of seed (mg.).	No. of primordia 10 days after beginning vernalisation treatment.
10 days	1/10 standard	2·5	2·7
10 days	Standard	3·4	2·8
20 days	1/10 standard	10·0	3·2
20 days	Standard	10·0	3·4
Mature spring rye	Standard	25·0	4·0

In 1949 seed was harvested either 10 or 20 days after anthesis, from plants grown with either standard nitrogen supply or 1/10 standard since there was evidence that seeds matured more slowly when the nitrogen supply was low. This grain was vernalised in sand with unrestricted moisture for 57 days at 2–4° C. under continuous light: care was taken not to cover the grain with sand during this treatment. Three sets of controls were grown, using seed harvested 20 days after anthesis from parents grown with low nitrogen supply: (1) vernalised in darkness; (2) unvernalised, germinated at room temperature in light; and (3) unvernalised, germinated at room temperature in darkness. The mean grain weights used and the number of primordia (determined in samples taken 10 days after vernalisation had begun) are given in Table XI.

All the grain was planted in sand culture under continuous light in the open on May 11. The condition of the apex of these plants is recorded in Table XII.

The lowest recorded leaf number for a single plant was 5; this occurred in 38 per cent. of the plants grown from vernalised grain harvested 20 days after anthesis from 'low nitrogen' parents. The *mean* leaf number of this series was 5·6: during growth 2·4 new leaves were thus added to the number (3·2, Table XI) in the grain. No lower number has yet been recorded for Petkus rye. The various effects that may be determined from Table XII are those of (1) maturity of the grain at harvest, (2) nitrogen supply to parent plants, and (3) illumination during vernalisation treatment.

Age at harvest had little effect on leaf number in winter rye. A low nitrogen supply to the parent plant reduced the final leaf number in the next generation to a degree which, though small, was significant for the grain harvested 20 days after anthesis. The score was also reduced: the magnitude of both leaf number and score, however, change in the same sense; the effect is then more likely to be related to vegetative growth. Illumination during vernalisation slightly but significantly increased the score and reduced the leaf number.

TABLE XII

Response of Prematurely Harvested Winter Rye Grain to 57 Days' Vernalisation in either Continuous Light or in Darkness. Planted May 11, 1950, in Continuous Light at 1/10 Standard Nitrogen Supply

	Vernalisation.			Condition of plants 19 weeks after planting.					
Maturity of grain.	Weeks.	Illumination.	N. supply to parent.	Score.	Final leaf No.	New leaves formed.	Apex length in mm.	No. of spikelet pairs.	Replicates.
10 days	8	Light	Standard	131·3	5·8±0·07	3·0	23·5	5·9	61
10 days	8	,,	1/10 standard	129·8	5·7±0·06	2·9	21·1	4·5	60
20 days	8	,,	Standard	137·2±0·4	5·9±0·06	2·5	26·5	7·5	48
20 days	8	,,	1/10 standard	136·8±0·3	5·6±0·06	2·4	25·7	7·0	60
20 days	8	Dark	1/10 standard	132·1±0·3	6·4±0·08	3·2	30·1	10·1	48
20 days	0	Light	1/10 standard	54·4	17·6	14·4	54·8	25·0	47

The significant point emerging from this experiment is that although the use of immature grain reduced the leaf number, the reduction does not parallel the reduction in the number of primordia in the grain: that is to say that rather more additional leaf primordia were laid down after imbibition when the initial number was low. Thus the concept of a minimal leaf number may still stand.

i. Treatment of a single leaf with continuous light

In both 1949 and 1950 single leaves of rye plants were exposed to continuous light in order to determine if the effect could be transmitted to the rest of the plant maintained under short-day conditions. In 1949 the results were inconclusive, but in 1950 definitive evidence of the transmission of the effect was obtained. The illumination of a single leaf presented some difficulty which, in cereals, is enhanced by the structure of the leaf sheath.

Apparatus. An apparatus was constructed to meet as far as possible these difficulties and is shown in Fig. 7. The plants were grown in water culture (Crone's solution was found suitable for this purpose), the solution was held in a rectangular tank covered by a sheet of duralumin through which, at 6-in. intervals, passed twelve open glass tubes 1 in. diameter; these were held in place by adjustable rubber rings. The plants were supported in these tubes by split corks, so that their roots were in the tank of solution and their leaves above the duralumin sheet. On this duralumin base was built an open wooden box having slots in its long walls opposite each plant position. Each slot could be closed by two shutters running in light-tight grooves and meeting in any desired position. The single leaf to be treated passed out through the slot and was held in place by the shutters, which were fitted with light-tight edges of black velvet. During the dark period of 10-hour days the box was darkened by a cover of black rubberized cloth which could be fastened under a projecting ledge, leaving only the treated leaves in continuous light.

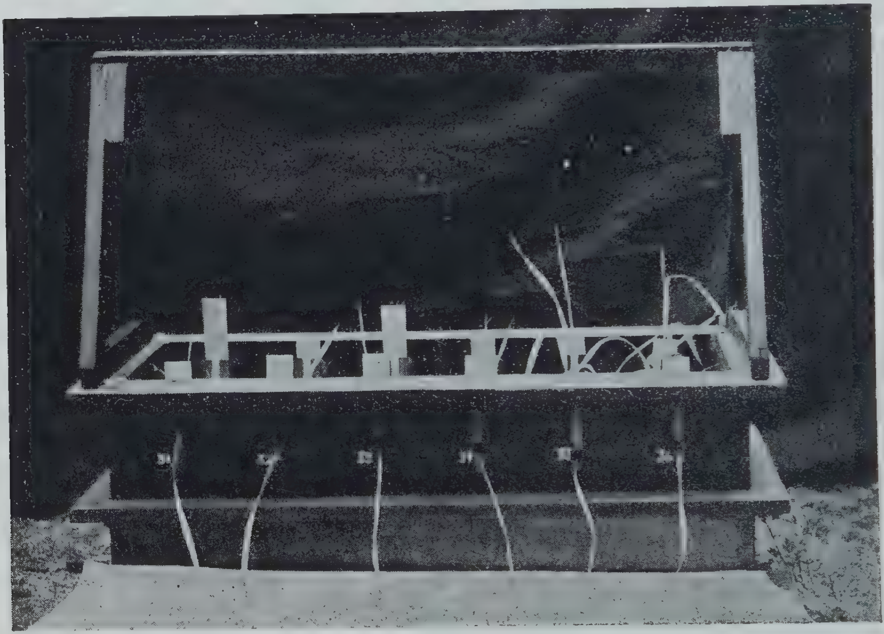

FIG. 7. Apparatus used for exposing single leaves of plants to continuous light, the remainder of the plants being kept in short days. For description see text.

In each tube two plants were grown, one of which, entirely in short days, served as a control to the other which had one leaf exposed to continuous light. The 14 hours of extra light received by these treated leaves was made up in part of sunlight and in part of artificial light of 20–25 f.c. Three such boxes were used, accommodating 36 treated and 36 control plants.

Method. Fully vernalised plants (11 weeks) and unvernalised plants were treated in this way, but results for the unvernalised plants were inconclusive, and those for vernalised plants only will be reported. The treated leaf was usually the youngest in which the ligule was fully exposed; it was held in position until senescence was apparent, when a younger leaf was substituted. Of the treated leaf a small portion of the lamina and the whole of the leaf sheath remained under short-day conditions, and a small permanently darkened portion of the lamina intervened below the blade which was in continuous light. In half the plants, whether treated or control, all other leaves except the youngest mature leaf were removed. In the remaining plants all leaves on the main axis were left intact: tillers, however, were removed from all plants as they appeared. Many plants died under this treatment and only one pair of defoliated plants survived, together with seven pairs of intact plants. Only those pairs of plants in which *both* partners remained in a healthy condition have been used in constructing Table XIII: the data were obtained by dissection 90 days after planting.

TABLE XIII

The Effect of Treating a Single Leaf with Continuous Light, the Rest of Plant remaining under 10-hour Day. Experiment of 1950. Plants vernalised 11 weeks, Standard Nitrogen Nutrition

Plant pair No.*	Score.			Leaf number.		
	Treated.	Control.	Difference.	Treated.	Control.	Difference.
1	35	31	+4	15	15	0
2	37	27	+10	10	17	−7
3	35	30	+5	11	17	−6
4	35	30	+5	12	18	−6
5	35	30	+3	13	15	−2
6	38	30	+8	12	17	−5
7	45	33	+12	12	16	−4
8	33	22	+11	11	16	−5
Mean	36·4	29·1	+7·25	12·0	16·4	−4·4

* Nos. 1–7, leaves intact; No. 8 defoliated except for a single treated or control leaf.

Plant pair No.*	Ear length in mm.			Total primordia.		
	Treated.	Control.	Difference.	Treated.	Control.	Difference.
1	11·0	6·5	+4·5	71	75	−4
2	52·0	3·2	+48·8	50	75	−15
3	12·0	3·7	+8·3	49	69	−20
4	12·0	4·0	+8·0	42	76	−34
5	4·5	3·4	+1·1	53	59	−6
6	68·0	4·5	+63·5	46	75	−29
7	82·0	8·5	+73·5	52	76	−24
8	5·2	0·9	+4·3	51	33	+18
Mean	30·8	4·3	+26·5	51·7	67·2	−15·5

* Nos. 1–7, leaves intact; No. 8 defoliated except for a single treated or control leaf.

Results. The treatment of a single leaf of a vernalised plant resulted in a higher score and a lower leaf number in every pair, and one treated plant eventually reached anthesis after the conclusion of the experiment, despite the short-day conditions under which the greater part of the plant was growing. The length of the ear is far ahead in the treated plants and they appear to have entered the 'grand period of growth'; the early cessation of apical growth is reflected in the reduced number of primordia. This does not apply to the defoliated pair (No. 8).

It may thus be concluded that continuous light has a direct accelerating effect on flowering transmitted from the leaf to the apex, which is not countered by a transmissible inhibiting substance produced in leaves exposed to short days.

k. Effect of interrupting the dark period on the photoperiodic induction of winter rye

It has already been pointed out that flower initiation in winter rye is to some extent earlier in short days than in natural long days and that in this respect winter rye behaves like a short-day plant. It is now well known that the accelerating effect of long dark periods on short-day plants is annulled by a light break in the middle of the dark period. In order therefore to determine if winter rye resembles other short-day plants in this respect, plants were submitted to the following treatments: (1) days of 17 hours throughout; (2) 10-hour days for 6 weeks, then transferred to 17-hour days; (3) 8-hour days for 6 weeks, then transferred to 17-hour days; (4) an interrupted dark period of 14 hours—thus a cycle of 9 hours daylight, 7 hours dark, 1 hour weak artificial light, 7 hours darkness—in all a total of 10 hours light per day for 6 weeks, then transferred to 17-hour days. Results of these treatments are given in Table XIV.

TABLE XIV

Effect on Photoperiodic Induction by Short Days caused by Interrupting the Dark Period. Short-day Treatment applied for 6 Weeks followed by 13 Weeks in 17-hour Days. Experiment of 1952. Planted May 1st. N. of Replicates

Daily light régime	Light 17 hrs. Dark 7 hrs.	Light 10 hrs. Dark 14 hrs.	Light 8 hrs. Dark 16 hrs.	Total light 10 hrs. Dark 14 hrs. interrupted by 1 hr. light at midnight.
'Score' at 19 weeks	26±1·7	39±3·5	42±5·2	26±1·5
Leaf number	21·3±0·4	18·6±0·4	18·9±0·7	21·2±0·3
Proportion past double ridges	13/17	13/14	16/17	13/20

The effect of the 1-hour interruption at midpoint of the dark period, shown in the last column, is to reduce the 'scores' and raise the leaf number to the level of plants in 17-hour days, thus showing that the photoperiodic induction effect of short-day treatment in flower acceleration is annulled by the light break.

DISCUSSION

To account for the relation between vernalisation and photoperiodic reactions in the development of the rye plant the following formal scheme has been proposed. The stages of elaboration of this scheme and the relevant experimental data are presented in the following papers: Studies in vernalisation of cereals, P. & G. I (1937); G. & P. II (1938); and P. & G. XII (1952).

$$\begin{tikzcd}
A \arrow[r, shift left] & A^1 \arrow[l, shift left] \arrow[r] & B \arrow[r, shift left] \arrow[d] & C \arrow[l, shift left] \arrow[r] & D \arrow[r] & {} \\
 & & E & & &
\end{tikzcd}$$

The reactions A to B are related to temperature effects during the vernalisation treatment and are not the concern of this paper; suffice it to say that A is the precursor of the vernalisation 'substance'; A^1 is a thermolabile intermediate which as vernalisation progresses is converted to a thermostable product B. In the embryo of spring rye B is already present; its formation is determined by a genetic factor (Purvis, Studies V, 1939) and is independent of the action of low temperature. In both spring and winter rye B is assumed to increase autocatalytically (Purvis, Studies XI). All development beyond B is mediated by photoperiodic reactions.

Reaction $B \rightleftarrows C$. This leads to the production of the precursor of a flower-initiating substance. This reaction is assumed to progress more rapidly in short than in long days. By an alternative path B can be diverted to the formation of E, which is not concerned in flower initiation but may be a leaf-promoting substance. The reason for assuming that the reaction $B \rightleftarrows C$ is reversible has been discussed in a previous paper (P. & G. I, 1937) and derives from the inductive effect of short days given during the first 6 to 8 weeks' growth of unvernalised winter rye. This reaction $B \rightarrow C$ progresses most rapidly in darkness and is partially inhibited by light. Owing to the reversible nature of the reaction, B and C tend to reach an equilibrium. If therefore the further reaction $C \rightarrow D$ is prevented, both C and B are depleted by the reaction $B \rightarrow E$. That the reaction $B \rightleftarrows C$ is not completely arrested by light is deduced from the fact that even when grown in continuous light unvernalised winter rye proceeds to flower initiation. Nevertheless, that photo-induction of unvernalised winter rye is possible indicates that in the stage preceding flower initiation unvernalised winter rye behaves like a short-day plant. This is confirmed by the effect of interrupting the dark period at its midpoint by exposure to light (Table XIV) which prevents photoperiodic induction. Unvernalised winter rye thus behaves like a facultative short-day plant.

Reaction $C \rightarrow D$. The stage C is the substrate for a further reaction $C \rightarrow D$ which proceeds only in light and is irreversible. 'C is responsible for early stages in flower initiation' (P. & G., I, 1938, p. 587). The reaction $C \rightarrow D$ may be assumed to take place in the 'labile primordia' and as D accumulates to a threshold value the process of flower initiation becomes manifest by the production of 'double ridges' and the development of the spikelet primordium in the axil of the bract. For a time, therefore, both C and D must be present, but the faster the reaction $C \rightarrow D$ proceeds the higher the relative amount of D will become. As the development of the spikelet primordium advances the bract primordium is gradually obliterated (Fig. 2) and in the mature ear is found only as a mere vestige in the basal regions. In the labile or 'physiologically double' primordia both C and D are present and if the accumulation of D fails to reach the threshold value the primordium remains a 'simple ridge'. Owing to the reversible reaction $B \rightleftarrows C$ and the side reaction $B \rightarrow E$, short-day conditions, which hinder the reaction $C \rightarrow D$, lead to the development of the 'simple ridge' into a foliage leaf. For this reason unvernalised winter rye kept in short days beyond the optimal period for short-day induction goes on producing leaves, whereas transfer to long days or continuous light at this stage results in spikelet production (P. & G. II, 1938). The reactions beyond D are also light dependent and result in further development of floral organs up to complete spikelet formation.

The photoperiodic reactions thus comprise two stages; the first leading to initiation, the second to the production of double ridges and further floral development.

Apical growth

The stage of initiation is associated with a rapid increase in the relative growth rate of the ear (Purvis, 1934) except in short days, and the beginning of the grand period of growth of the apex which terminates when the ear is fully developed.

Although the rapid growth of the apex is associated with double-ridge formation, these processes are independent. Thus in unvernalised winter rye in short days double-ridge formation occurs after 12 weeks yet the relative growth rate of the apex remains constant up to 19 weeks (Fig. 5), whereas in continuous light double-ridge formation occurs after 8 weeks and is associated with a rapid increase in growth rate of the apex (Fig. 4*a*). In spring rye in short days double ridges appear in 7 weeks but no increase in growth rate of the apex follows (Fig. 5), while in fully vernalised winter rye and in spring rye in continuous light the very early appearance of double ridges after 3 weeks (Table VI) is preceded by the beginning of the grand period which occurs 1 week from planting out. The interaction of degree of vernalisation and daylength is seen in Fig. 4*a*, *b*; with short periods of vernalisation the difference between long day and continuous light is very apparent, but with longer vernalisation treatment the differential effect of these daylengths is much reduced.

Before flower initiation begins the relative growth rate of the apex remains constant (Figs. 4*a*, *b*, 5), but daylength has an effect, as the steady relative growth rate in continuous light is always higher than in normal day.

In short days during the early stages (photo-inductive phase) the relative growth rate is higher than in long days, but lower than in continuous light (Fig. 3, 1953 expt.); both short days and continuous light thus lead to accelerated growth of the apex. The short-day acceleration of apical growth is, however, dependent upon an adequate supply of nitrogen (Fig. 3; cf. 1950, 1953 expt.).

Grand period of growth of the apex after flower initiation

The effect of light is also seen after initiation of flowering has begun. In continuous light the rate of growth is higher than in long days in all treatments given *less* than 3 weeks of vernalisation (Fig. 4*a*, *b*), and over the range of vernalisation there is a marked interaction between daylength and duration of vernalisation. In short days (Fig. 5) no grand period of growth appears either in spring rye or unvernalised winter rye; in long days the growth rate in the grand period increases with duration of vernalisation and is scarcely apparent in unvernalised winter rye; while in continuous light the grand period is well marked even in unvernalised plants.

The rate of growth after flower initiation has begun is thus clearly dependent on the level of B in the apex. Leaving aside for the moment the question of the active agent in promoting the growth of the apical meristem, the question of flower initiation may next be considered.

Relation of flower initiation to daylength and vernalisation

The data in the following table summarize the results obtained in three seasons and show the effect of daylength on time to double-ridge formation in spring rye or fully vernalised winter rye and unvernalised winter rye.

TABLE XV

Relation of Daylength and Time to Double-ridge Formation in Spring or Fully Vernalised Winter Rye and Unvernalised Winter Rye

	Daylength.	Weeks to double-ridge formation.		
		1950	1952	1953
Spring rye and fully vernalised winter rye	24 hrs.	3	—	—
	Normal L.D.	3	—	—
	S.D. 10 hrs.	7	—	—
Unvernalised winter rye	24 hrs.	8	—	< 7½
	Normal L.D.	15	10	10
	S.D. 10 hrs.	12	8	—
	S.D. 8 hrs.	—	6	7½

The preponderate accelerating effect of degree of vernalisation on flower initiation is evident, and the retarding effect of short days. In normal daylength the differential effect on vernalised and unvernalised rye is apparent; short days in all three seasons lead to more rapid initiation of unvernalised plants than normal long days (photo-inductive effect of short days). Evidently the time to initiation is dependent upon the level of B. The two reactions $B \rightarrow D$, $B \rightarrow E$ determine the partitioning of B and the formation of flower and leaf stimulating substances. Since it has been assumed that double-ridge formation occurs at a definite threshold level of D, the data in the table refer to a comparable stage in the reaction chain. It may be permissible from the data of 1950 to attempt an evaluation of the rates of production of D and E in relation to the factors studied. For this purpose the relative rate of *D* production may be estimated from the reciprocal of the values in column 3, and the rate of *E* production from the rate of production of leaves (Tables IV and V). These derived data are entered in the following table, and also the relative rates of *E* and *D* production.

TABLE XVI

Effect of Vernalisation on the Rate of Production of D and E

	Daylength.	Rate of *D* production.	Rate of *E* production.	Relative rate $E/D \times 10^{-1}$.
Spring rye	24 hrs.	0·33	2·0	6·1
	L.D.	0·33	1·9	5·8
	10 hrs.	0·14	1·6	11·4
Mean	—	0·27	—	7·8
Unvernalised winter rye	24 hrs.	0·125	1·2	9·6
	L.D.	0·067	1·2	18·0
	10 hrs.	0·083	1·2	14·5
Mean	—	0·092	—	14·0

Rate of *D* production = Reciprocal of time in weeks to 'double ridges'.
Rate of *E* production = Final number of leaves/Time in weeks to reach maximal leaf number, i.e. rate of leaf production per week.

The high level of *B* in spring rye as compared with unvernalised winter rye is reflected in the rate of both *D* and *E* production as would be expected, but the effect of level of *B* is much less marked on rate of *E* production. Continuous light in both cases increases the rate of *D* relative to *E*, and this may be regarded as due to the more rapid rate of reaction $C \rightarrow D$. In spring rye the reduction of daylength to the normal natural daylength has little effect, but on unvernalised winter rye the effect is large, and is due to the reduction of formation of *D*. In this case the rate of production of *E* is not affected, and it may be assumed that the rate of this process is limited by some other factor. In short days the rate of *D* production is increased over that in long days in unvernalised winter rye and decreased in spring rye. It may therefore be concluded that the acceleration of $B \rightarrow C$ by short days in unvernalised winter rye more than compensates for the slower rate of reaction $C \rightarrow D$ than in normal daylength. The slow rate of reaction $C \rightarrow D$ in short days is reflected in the behaviour of spring rye in which the relative rates of the two reactions, $B \rightarrow D$, $B \rightarrow E$, is greatly increased in favour of *E* formation.

The interaction of daylength and vernalisation on the rate of primordium formation subsequent to initiation

From the data of 1950 the rate of differentiation of primordia in the apex after initiation has been assessed; the difference between primordial number at initiation and the final number of primordia is divided by the number of weeks from initiation (beginning of the grand period of growth) to the time at which final primordial number is reached.

The results are entered in the table below.

TABLE XVII

Interaction of Vernalisation and Daylength on the Rate of Primordia Formation after Initiation

	Day-length.	No. of primordia			Time to reach max. in weeks.	Rate of primordia formation.
		At initiation.	Final.	Increase.		
Spring rye	C.L.	7·6	18·7	11·1	1	11·1 (100)
	L.D.	6·4	23·3	16·9	2	8·5 (77)
	S.D.	12·2	50·2	38·0	10	3·8 (35)
Mean	—	—	—	—	—	7·8
Unvernalised winter rye	C.L.	16·5	43·1	26·6	6	4·4 (100)
	L.D.	24·8	46·5	21·7	7	3·1 (70)
	S.D.	24·0	41·3	17·3	9	1·9 (43)
Mean	—	—	—	—	—	3·1

The main effect of vernalisation is to increase the rate of primordia formation by a factor 2·5. This is thus an estimate of the relative concentrations of *D* in the apex, and this may be compared with the values of 2·9 derived from the mean rates of *D* production in Table XVI, in this case referring to the time for threshold value in double-ridge formation; the agreement is as close as the data would warrant.

It is further seen that the relative effect of daylength is similar in the vernalised and unvernalised plants as the data in brackets in the last column show. There would thus appear to be no interaction in this case between vernalisation and daylength. As the reduction in daylength between the treatments is in the ratio 100:71:42 while the reduction in rate of primordium formation is 100:73·5:39, it is clear that the whole effect is due to the hours of daylight, which confirms the suggestion that all reactions beyond *D*, &c., are dependent entirely on daylength, and that the rye plant after induction is a true long-day plant.

The relation of induction to leaf number

It has been assumed in past papers that the stage of 'ripeness to flower' is associated with attainment of a minimum leaf number. The very early initiation in spring rye or fully vernalised winter rye suggested the possibility that the vernalised grain is already 'ripe to flower', and that exposure of the green leaf to light sets in train the photoperiodic reactions leading to flower initiation. As the fully vernalised embryo has 4 primordia already present, and since the lowest leaf number found in continuous light was as low as 5, it seemed possible that the obligate vegetative phase is absent in the rye plant. To test this use was made of prematurely harvested grain with 'dwarf embryos' which contained as few as two primordia (Table XI). When such grain was vernalised in continuous light and thus given the most favourable conditions for initiation it was found that three extra primordia were produced before initiation and the final leaf number remained at 5·8 (Table XII). It would thus appear that the notion of minimal leaf number is valid, and has the value of 5.

The behaviour of the labile primordia

The primordia after the 5th are termed 'labile' or 'physiologically double' as they may eventually give rise to spikelets or foliage leaves according to the conditions of daylength. The final leaf numbers for 1950 were as follows:

	C.L. (24 hrs.).	Normal day.	S.D. (10 hrs.).
Fully vernalised (spring rye)	6·6	7·6	16·0
Unvernalised	17·2	22·1	21·8

The number of 'determined leaves' at the time of double-ridge formation for the same series of treatment was as follows:

	C.L. (24 hrs.).	Normal day.	S.D. (10 hrs.).
Fully vernalised	6·9	7·3	12·9
Unvernalised	14·0	19·8	15·4

It is clear that in fully vernalised and spring rye both in continuous light and normal day all labile primordia after initiation become spikelets, but in short days 3·1 of the primordia present on the apex eventually become foliage leaves. In unvernalised winter rye, under all daylength conditions, some of the labile primordia become foliage leaves, the largest number 6·4 again in short days.

Double-ridge formation occurs not in the lowest primordia destined to become one of the spikelets of the ear, but in a position which eventually is central on the ear. There is good evidence (Purvis, 1940) that the location of *B* is in the apical meristem, and thus a stream of *B* is translocated in a basal direction and a gradient established. The primordia situated above the point of 'double-ridge' formation are still too immature to respond, and those below deficient in *B* at this time.

In the particular primordium in which double ridges appear the level of *D* has reached the threshold value. From the fact that eventually even in continuous short days spikelet formation occurs in unvernalised winter rye it is assumed that the level of *D* rises in each successive primordium formed on the apex (by autocatalysis of *B*), so that the primordia below constitute a series of descending level of *D*. In continuous light the conversion of $C \rightarrow D$ is so fast that the labile primordia contain little or no *C*, and in consequence all the primordia become spikelets as soon as the 'minimum' number of vegetative primordia have been laid down and the photoperiodic reactions can begin. This is so in the fully vernalised or spring rye where the level of B is high; in unvernalised winter rye where the level of B is necessarily lower the threshold value of D is not reached immediately after the minimal number of plastochrones have elapsed, and therefore even in continuous light a few extra foliage leaves are formed.

Once the threshold value of D is attained in any primordium, spikelet formation proceeds irreversibly, but in the primordia below, both C and D are present in varying proportions according to daylength conditions, i.e. according to the rate of reaction $C \rightarrow D$. If the conditions are unfavourable for D formation so long as C is present the reversible reaction $C \rightarrow B \rightarrow E$ is still possible and thus to a limited degree the 'bract' primordium may develop, and, thus, the abnormal case arises that the spikelet is subtended by a much reduced leaf as is seen in Fig. 6 and Table IX. As Wycherley (1952, 1954) has shown, this may occur more readily in *Cynosurus cristatus*.

Spikelet production in the ear

The effects of daylength on the development of the ear are twofold: (1) on the growth rate of the apex after initiation, (2) on the number of spikelet pairs produced. The relation between these factors is summarized in the following table. The relative growth rate entered is the logarithmic increase in apex length over the period from onset of the grand period of growth (in case of short days the formation of double ridges) to the time of full ear development.

TABLE XVIII

Interaction of Daylength and Vernalisation on Growth of the Ear and Spikelet Number

	Day-length.	Relative growth rate of ear.	Spikelet pairs.	Ratio × 10^{-2}
Unvernalised winter rye	C.L.	0·21	25·9	1·2
	S.D.	0·05	19·5	3·9
Spring rye	C.L.	0·12	11·1	0·9
	S.D.	0·11	34·2	3·1

The data in the table show that the relation between the number of spikelets and the growth rate of the apex is almost independent of the degree of vernalisation (Mean spikelet number). On the other hand, a strong interaction of growth rate and degree of vernalisation appears: for in spring rye (or fully vernalised winter rye) short day *v.* continuous light has little effect on the growth rate, while in unvernalised winter rye daylength has a large differential effect on the growth rate. Daylength affects the relationship of spikelet number and growth rate to the same degree in vernalised or unvernalised plants; the factor for continuous light *v.* short day being 3·4 in spring rye and 3·3 in unvernalised winter rye. The number of spikelets for a given growth rate of the apex is three times greater in short days than in continuous light; this again suggests that the factors controlling primordial formation and apical growth are distinct.

The relation between spikelet number and daylength has been fully investigated by Hänsel (1951) using vernalised Petkus winter rye. The grain was vernalised for 76 days and then grown in short days of 8 hours for varying number of weeks before transference to long days. Up to 5 weeks of preliminary short-day treatments the spikelet number increased in a linear manner with duration of short-day treatment; the values obtained were 19·2 spikelets in long days throughout and 31·4 after 5 weeks' preliminary short days followed by transfer to long days. Grown throughout in short days the spikelet number increased further only slightly to 39·5. Evidently the production of labile primordia continues at a constant rate in short days, and that after transfer to long days further production of primordia ceases and is replaced by spikelet development.

In continuous light the reactions beyond D proceed so fast that the concentration of B is kept to a minimum by the reactions $B \rightarrow C \rightarrow D$ and thus the production of E is also minimal. E may be concerned in the production of 'simple primordia' and thus in continuous light primordium production in the apex is decreased, and there is no locus present for the flower initiating action of C. In short days, however, the reaction $C \rightarrow D$ is hindered and thus more of B is available for E formation, and as the level of B increases by autocatalysis these primordia eventually become spikelets even though the reaction $C \rightarrow D$ is slowed down by short light exposure.

The factors controlling apical growth and flower formation

There is evidence to show that the factors controlling primordium formation and apical growth are distinct.

Since growth is intimately associated with auxin production, evidence for an auxin mechanism needs consideration. The mere fact that extension of the stem internodes follows upon flower initiation would suggest some causal relationship with auxin production. An extensive investigation of auxin production in the apex of winter rye submitted to various treatments of daylength and vernalisation has been carried out and will be published in due course. It has been established that there is a close relation between 'score' in various treatments and auxin content of the apex, but that no marked change in auxin production in the apex occurs at the time of double-ridge formation.

Comparing auxin content of the ears of vernalised and unvernalised plants grown in continuous light showed no consistent differences when ears of comparable score (up to ear emergence) were compared, though the mean growth rate of the vernalised is only half that of the unvernalised ear. Nutritive factors certainly play a part. Thus the growth rate of the apex is increased by nitrogen application, and progress to flowering is hastened (Table I*b*). Carbohydrate supply may also contribute to the daylength effect.

The work of Leopold and Thimann (1949) with Wintex barley is relevant in this connexion. They produced evidence to show that auxin (IAA or NAA) in low concentration supplied to the plant increased the number of floral primordia laid down. Repetition of this work using Petkus winter rye showed no effect either on initiation or number of flower primordia produced, and with Wintex barley no effect on time of flower initiation was observed, although a transient effect on the growth of the apex after initiation was found in one out of four experiments (Hussey and Gregory, 1954). The role of 'auxin' thus appears to be secondary. In so far as auxin is required for the processes of cell multiplication at the apex to continue, the part it may play in the development of the apex is predictable. There may be active competition for available auxin between the developing flower primordia and the terminal meristem of the ear; if so, the termination of growth and the reduced number of spikelets found in the ear of spring and fully vernalised rye in continuous light as compared with short days may be explained. There is, however, a further effect of vernalisation superimposed on the daylength effect. The spikelet number, as already pointed out, bears a constant relation to the growth rate of the apex; and the small number of spikelets obtained in short days with unvernalised winter rye is due to the low relative growth rate. The relative growth rate of the ear in continuous light is twice as great in unvernalised as in vernalised rye; in short days the reverse holds, vernalised being twice that of unvernalised (Table XVIII). As already stated, preliminary work on the auxin content of the ear shows no marked effect of daylength: the cause of the differential effect is thus at present unknown.

The final point needing discussion is the effect of continuous light given to a single leaf of plants otherwise exposed to short days. All characteristics of the plants—increased score, reduced leaf number, ear length, and total primordia (Table XIII)—show that photoperiodic effects can be transmitted from the illuminated leaf. Clearly vernalised winter rye behaves like a long-day plant in this respect.

It has been assumed that the observed photoperiodic effects are due to reactions occurring in the apex, and further the scheme presented will not accommodate any 'substance' entering the system in response to daylength conditions. It may be necessary to modify and further elaborate the scheme to meet this possibility. It may indeed be held that the data support the view of Melchers and Lang (1948) of two separate 'substances', 'vernalin' and 'florigen', which interact to produce the flower hormone. The question will be left in abeyance until further experiments on transmission of photoperiodic effects have been carried out. In other respects the scheme will adequately account for the phenomena of flowering in relation to low temperature, daylength, and their interaction.

SUMMARY

The following are the more important results obtained.

1. Progress to flowering is unaffected by nitrogen level in highly vernalised plants; in unvernalised and slightly vernalised plants nitrogen deficiency retards progress to flowering.

2. In *fully vernalised* winter rye flower initiation is complete 3 weeks after planting out in continuous light (C.L.); in normal days (L.D.) full initiation is delayed by 1 week, in short days (S.D.) initiation occurs after 6–7 weeks. In *unvernalised* winter rye floral primordia appear in 8 weeks in C.L., 12 weeks in S.D., and 15 weeks in L.D.

3. The ear is fully determined in spring rye after 3 weeks in C.L., 4 weeks in L.D., and 14 weeks in S.D., the number of spikelet pairs being 11·1, 15·7, 34·2 respectively. In unvernalised winter rye ear determination is complete in 12 weeks in C.L., but development continues for 19 weeks in L.D. and S.D., the final numbers being 25·9, 24·4, and 19·5 respectively.

4. Unvernalised winter rye in C.L. maintains a constant relative growth rate of the apex for 8 weeks up to initiation; in L.D. the relative growth rate is constant for 12 weeks but at a lower level. In S.D. relative growth rate of the apex is constant for 19 weeks, and with adequate nitrogen supply the growth rate in S.D. is higher than in L.D.; in S.D. spring rye has consistently a higher relative growth rate of the apex than unvernalised winter rye.

5. In C.L. with all durations of vernalisation including no vernalisation the relative growth rate of the apex increases rapidly after initiation, the ear entering upon a grand period of growth. In L.D. the rate of growth after initiation increases with duration of vernalisation up to 3 weeks. In S.D. no grand period of growth follows upon initiation in any vernalisation treatment. The factors controlling initiation and subsequent ear development are thus distinct.

6. After initiation the rate of primordium formation in the apex depends upon the duration of vernalisation, but there is no interaction with daylength (Table XVII). After initiation the rye plant behaves as a true long-day plant; the rate of primordium formation is proportional to the number of hours of light received.

7. Before initiation winter rye behaves as a facultative short-day plant displaying photoperiodic induction by S.D. which effect is annulled by interruption of the dark period by light of low intensity (Table XIV).

8. Using immature grain with dwarf embryos with as few as two primordia and vernalising in continuous light, it is shown that the final leaf number in C.L. remains at 5·8, similar to that of normal embryos with originally four primordia. An obligatory vegetative phase thus precedes 'ripeness to flower' which is attained at a minimal leaf number of 5.

9. Spring rye plants exposed to L.D. for 2 weeks and then transferred to S.D. showed abnormalities in the ear with the lower spikelets subtended by green leaves, spikelets replaced by secondary spikes, and extension of the spike internodes. This is regarded as evidence of partial reversion to the vegetative condition by reversal of one of the stages of the reactions leading to flower initiation. Transfer from S.D. to L.D. always results in normal ear development.

10. Treatment of a single leaf on a vernalised plant with C.L., the re-

mainder of the plant being kept in S.D., leads to an accelerating effect on flowering and reduction in leaf number, the plant thus behaving like a long-day plant.

11. The factors controlling primordium formation and apical growth are distinct. The relation of 'auxin' to growth initiation is discussed.

12. The discussion deals with the interpretation of these results in terms of the schema presented in earlier papers, and the relative rates of the various stages in the reaction chain are assessed.

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Studies in the Regeneration of Horseradish

I. A Re-examination of the Morphology and Anatomy of Regeneration[1]

BY

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With two Figures in the Text.

ABSTRACT

1. Horseradish roots reach a stage of 'ripeness to regenerate' at the age of 1–2 months, when secondary thickening has begun, a phellogen has formed, and the primary cortex has been shed; provided that they are then detached from the parent plant.

2. New primordia arise (as is already known) only in association with the scars of lateral roots; but they are produced, not as previously believed from a callus, but individually as organized meristems whose cells are derived only from the phellogen of the main root.

3. The primordia appear to be undetermined ('indifferent') in nature; their subsequent differentiation into buds or roots is described.

1. INTRODUCTION

REGENERATION in its widest sense has been defined (Vöchting, 1878; Sachs, 1887; Goebel, 1900; Morgan, 1901; Klebs, 1902) as the growth of new parts of a plant after the removal of corresponding parts elsewhere in the plant, a definition which can usefully be applied to the development of endogenous buds on the roots of horseradish (*Armoracia rusticana* Gaertn., Mey. & Scherb.). Such root-buds have been described for many plants (*vide*, e.g., Karsten, 1861; Beijerinck, 1887; Priestley & Swingle, 1929; Lindner, 1939; Emery, 1954), though in no case has a systematic investigation of the morphology and physiology of development been undertaken. Moreover, previous workers (e.g. Lindner, 1939) who have used horseradish root as material for investigating this problem have often hampered themselves by using large, old roots. Such roots have several disadvantages: first, the amount of secondary thickening is such that anatomical investigation is made unnecessarily complicated; secondly, many old roots will already have regenerated before being used as experimental materials; and finally, age is an important factor in the regeneration phenomena, and this cannot be accurately determined in very old specimens. The present investigation has therefore been carried out almost entirely on young roots regenerating for the first time.

This paper will deal only with the morphology and anatomy of the process; the effect upon it of certain environmental conditions and the associated

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correlation phenomena (which, involving as they must considerations of initiation, differentiation, and extension, are complex and sometimes novel) will be the subject of later communications.

2. MATERIAL AND METHODS

All the observations described below have been made on 2-cm.-long cuttings of horseradish root of thickness varying from 1 to 5 mm. At this stage of growth roots do not show excessive secondary thickening and are easy to examine and manipulate. The cuttings were arranged horizontally on damp sand in Petri dishes, five or six lengths being placed in each dish. In order to permit recognition of the proximal and distal ends of the cuttings they were arranged on the sand so that the proximal end always lay nearest the perimeter of the dish.

Anatomical observations were made on microtomed longitudinal sections, which were prepared from daily samples of root lengths, fixed in formalin-acetic-alcohol fixative, and embedded in wax by the usual techniques. Sections of 6 or 12 μ thickness were cut and stained with Delafield's haematoxylin or with haematoxylin and safranin by the usual methods, then mounted in 'Euparal'. The course of the vascular tissue was traced by staining hand sections in ammoniacal basic fuchsin. Finally, sections were drawn with the aid of a microprojector.

3. THE MORPHOLOGY OF REGENERATION

(*a*) *Preliminary considerations*

Lindner (1939) has reported that the primary root tissues possess three apical histogens, and confirmed Beijerinck's (1887) observations that the primary vascular tissue is diarch to pentarch. He further remarks that the pericycle remains as a single layer until immediately prior to cambium formation, when a multilayered region is formed. Primary lateral roots arise normally in the pericycle. The final stage of development is the formation of a cork cambium in the outer region of the pericycle, the whole of the original cortex being then sloughed off. Any additional lateral organs formed after the sloughing off of the cortex arise only in association with the scars of the first-formed lateral roots. It is important to note that the mature root has tissue of pericyclic origin as its outer tissue, so that all buds and roots arising from this tissue are physiologically endogenous in origin.

I have confirmed these observations of Lindner, and have in addition found (*a*) that the cortex will first show signs of breaking away from the root when the latter is just over 1 month old; and (*b*) that until the cortex has been shed the root will not regenerate at all.

(*b*) *New observations*

The newly formed root or bud normally arises singly alongside a primary lateral root scar. Occasionally two buds, or two roots, or one bud and one root, are formed beside a scar; if the members of such pairs arise simultaneously, observation shows that they are always formed by the division of a single meristem. The process is very rapid, the new primordium being visible to the naked eye in about 2 to 3 days after the root-cutting has been detached.

Lindner (1939) suggests that the buds and roots are formed on a callus. This is not so; the observations on the anatomy (see below) show that the first-formed regenerating buds and roots are each developed from a generalized primordium, which is organized with a well-defined layer structure. Buds can produce one or more true adventitious roots at their base, but regenerating roots have not been observed to produce adventitious buds, although these will often arise from a divided primordium, one half of which becomes a root; or buds may arise later from a separate primordium which develops near to a new root. As the numbers of buds and roots around a scar increase, each bud or root being formed from an individual primordium, there may be a swelling of the main root to accommodate each primordium. This swelling does not resemble callus and is due to the elongation and vacuolation of the oldest cells of the meristem.

Old thick roots sometimes have small tubercles surrounding or adjoining a lateral root scar, which have been found to form after the first-formed regenerating bud has been removed, and to consist of the swollen base of the bud. It is conceivable that, since Lindner was using old tap roots, swellings such as these were confused with a true unorganized callus; such tubercles sometimes form conspicuous rows along old tap roots. Emery (1954) has found that in *Chamaenerion* similar tubercles develop from the base of damaged buds. It is also to be noted that both regenerated buds and roots show a small 'neck' region separating the regenerating root or bud from the cutting. Beijerinck (1887) had noticed this and suggests that the lateral root or bud, separated thus, forms a true individual. No discernible function can be assigned to this region, which is less prominent in buds than in roots.

The limitation of available sites (i.e. root-scars) for regeneration may give rise to difficulties in the quantitative estimation of the degree of regeneration. The scars are sufficiently numerous and evenly spaced for 'primordia per unit length' to serve for all except very short cuttings. In the latter, or when particular accuracy is sought, it is preferable to count the scars and express the regeneration in terms of 'primordia per scar', a quantity which will normally vary between 0 and 1.

4. THE ANATOMY OF REGENERATION

(*a*) *Development of primordium*

Preliminary observations which were made on the anatomy of regenerating buds suggested that the observations made by Lindner (1939) were not wholly correct. He stated that the regenerating organs are derived from both the main root cork cambium and the lateral root vascular cambium. This has not been confirmed; and I hope to demonstrate that the new primordia

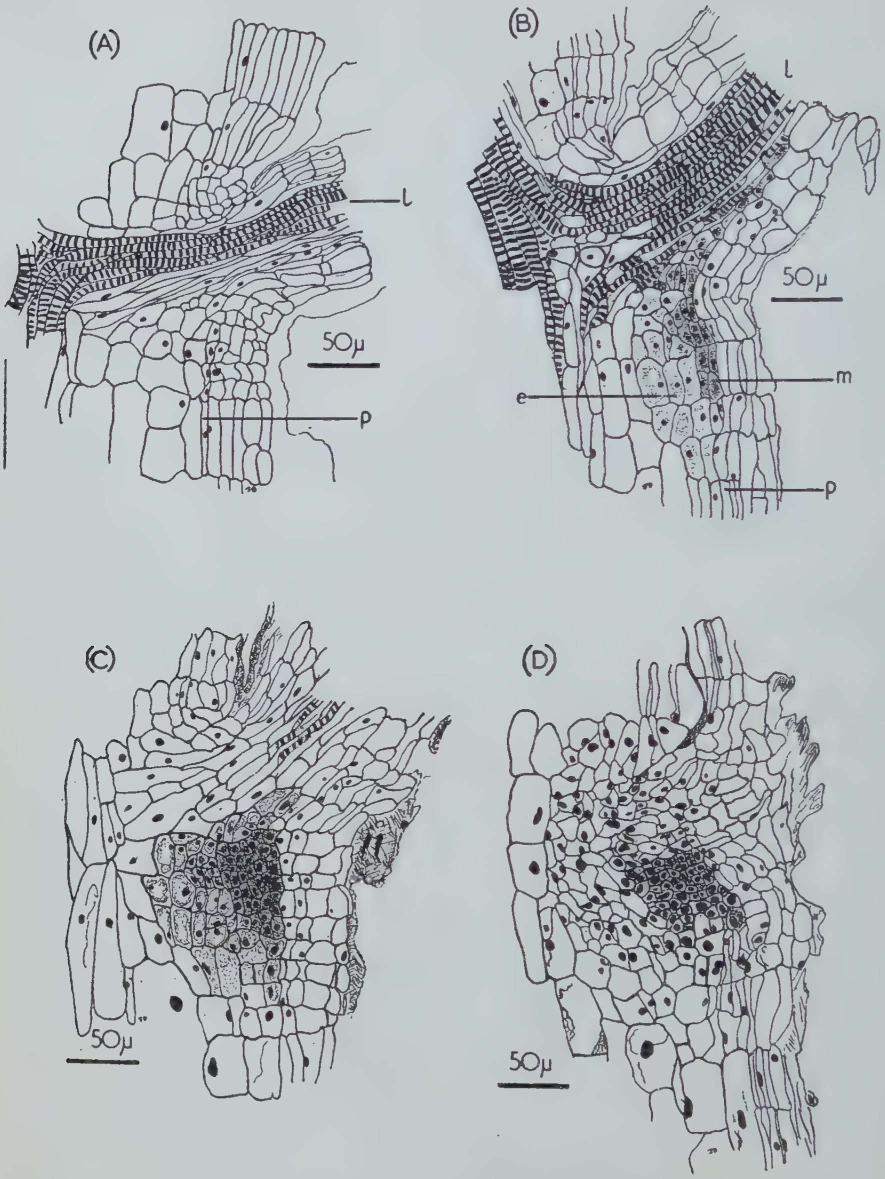

FIG. 1. Primordium development in horseradish. (*a*) L.S. through main root at lateral root (*l*) junction; (*b*) earliest stage in primordium formation: *m*, meristem; *e*, elongating zone; *p*, phellogen; (*c*) a later stage; (*d*) a stage comparable to (*c*) where the new meristem has arisen slightly lateral to the root-scar. For detailed explanation see text.

originate from a meristem produced by the cork cambium only, associated with the lateral root scar but having no cells from it.

A section through a typical lateral root (Fig. 1*a*) shows a distinct cork cambium surrounding both the main root and the lateral root. Remnants of the primary cortex are visible on the surface of the root. The vascular tissue of the lateral root leaves the main vascular system at right angles, as is usual: and, as will be seen later, this feature is retained by the vascular tissue of the regenerated organs. This stage, which shows no visible signs of primordium development, will, provided that environmental conditions are appropriate, within 2 days give rise to a fully developed primordium. The intervening stages thus succeed each other very rapidly, and consequently make it necessary to cut many sections to obtain a full record of development.

A small pad of meristematic cells develops from the cork cambium (Fig. 1*b*). It is associated intimately with the lateral root, but it is clear that the dividing cells are from the cork cambium and are not associated with the vascular cambium. Deep-seated tissues of the secondary cortex show some meristematic activity. The origin of the meristem is made clearer in Fig 1*c*. The cells of the cork cambium have given rise to parallel rows of cells which at this stage have become isodiametric, while, however, still retaining their relation to the cork cambium. A feature prominent in all sections is also shown clearly in Fig. 1*c*. It is the presence of a number of deep-seated cells with dense contents. These cells are apparently not dividing, but show the features (densely staining nuclei and dark contents) of active cells. These cells, which are part of the secondary cortex, will later elongate, so causing the meristem (which becomes organized into a primordium) to penetrate the cork.

A later stage (Fig. 1*d*) shows a young meristem which has arisen slightly lateral to the root-scar. Examination of other sections in series with the one drawn shows that the greatest extent of meristematic cells is visible in this section, and that there is no possible connexion between it and the vascular cambium of the lateral root. This section also shows, rather imperfectly, the continuity of the cork cambium with the primordium. The meristem has also grown in size, causing a slight rising of the main root surface, but no penetration of the cork. It is at this stage that the first signs of activity are visible on isolated lengths, i.e. normally 2 or 3 days after isolation of the cutting.

The organized primordium has by the end of 3 days penetrated the still functional cork cambium and the cork by an increase in the number of cells below the dividing meristem (Fig. 2*a*). These cells have been formed from the elongating primordium and cells of the cortex. There is no evidence in the figure, nor in any other section which has been examined, of any violent rupture of the cork. I conclude, therefore, that there is some solution of this tissue by the developing primordium. Even at this stage of growth there is no indication whether the primordium—for from its organized structure it is apparent that it is a primordium and not a callus—will develop into a bud or a root. Although there is a distinct layering of the primordium, pro-cambial strands are absent, and the meristematic region is not confined to the surface cells as in a bud, nor is there evidence of a root-cap or a deep-seated meristem. The development of such a primordium into a bud or root is described below.

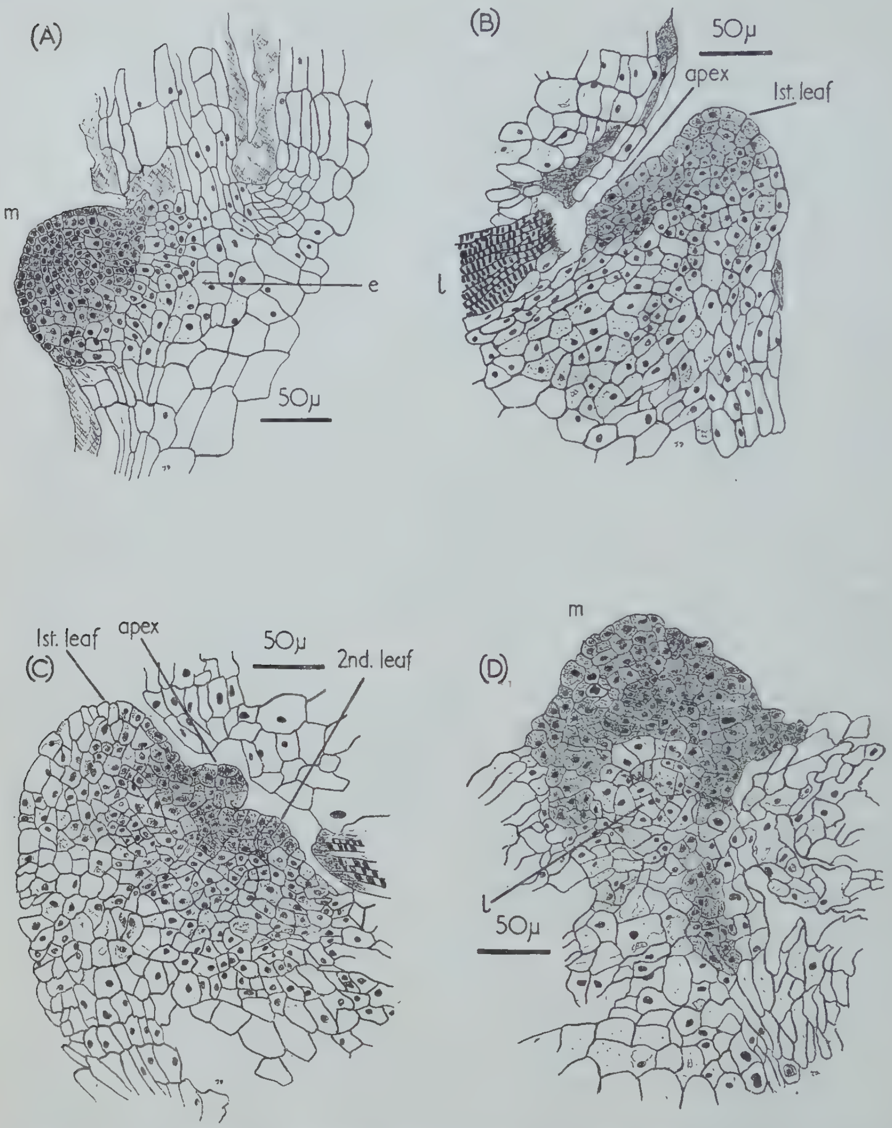

FIG. 2. Later stages in primordium development. (*a*) Primordium immediately before differentiation: *m*, meristem; *e*, elongating zone; (*b*) bud, 1-leaf stage; (*c*) bud, 2-leaf stage; (*d*) root. For detailed explanation see text.

(*b*) *Differentiation of a bud*

A bud is normally recognizable as such 2 days after the appearance of the primordium, i.e. 5 days after isolation of the cutting. The first sign is the development of the first leaf primordium laterally on the meristem (Fig. 2*b*). The leaf primordium forms at the surface of the cutting, so that the bud apex is concealed from surface view. The young bud shows a tendency to arise at an angle to the cutting, which, in the particular section figured, is undoubtedly connected with the original position of the meristem. A sunken root-scar, giving an indentation in the surface of the main root, will cause a curvature of the cork cambium surrounding the scar. A bud arising at right angles to the cork will, in the region of the curved surface of the root, tend to arise at an angle to the cutting. The alignment is complicated by the fact that the young buds are, as usual, negatively geotropic.

Further development of the young bud is straightforward. The first and second leaves develop (Fig. 2*c*), leaving a small apex without layered structure, which does not elongate. The sections show that connexion between the primordium and lateral root cambium is absent, and in the two-leaf stage vascular and procambial tissue is also absent. In the three-leaf stage vascular elements (tracheides) differentiate in the elongating zone at the base of the bud. Observations on mature buds show that the vascular connexion between the bud and the stele of the cutting is vertical; the vascular system of the bud does not join that of the lateral root, an observation which confirms that of Beijerinck (1887).

(*c*) *Differentiation of a root*

A root is normally recognizable as such 4 to 5 days after the appearance of the primordium, i.e. 7 to 8 days after isolation of the cutting. The primordium grows out rapidly as a normal young root, with a conspicuous root-cap and an underlying meristem (a very early stage is shown in Fig. 2*d*). At the base of the root there develops a prominent swollen region, formed by the enlargement of cells of the main root tissue with the addition of the oldest cells of the new root meristem. This wider region (which, of course, develops *after* the primordium) may well have been mistaken by earlier workers for a callus. Beneath it again there is a marked constriction, the 'neck' referred to earlier.

5. FURTHER DEVELOPMENT OF REGENERATED BUDS

The development of regenerated buds has been followed to maturity, and the following observations are of interest:

(*a*) The buds, at an appropriate stage of development, can be detached from the parent root and grown separately; the precise age at which this is possible has not been ascertained, but is of the order of 1 month. Such detached buds rapidly produce a long, thin tap-root. Later development is noteworthy for the production of new large roots from near the base of the crown.

(*b*) If the buds are *not* detached, then, of those initiated, only one (rarely two) will develop into a crown with its associated tap-root. Other buds present on the original 2-cm. length decay, and after about 3 months the original root-cutting itself will completely decay.

(*c*) In common with earlier workers, I have observed the occasional production of dissected leaves. In these experiments they have been particularly associated with pot-binding, root damage, or transfer to a heated greenhouse, and one of the plants has been figured by Audus (1953, fig. 51); but the phenomenon is undoubtedly complex, and my observations add nothing to the findings of Njoku (1948) and Pound (1949).

(*d*) The first flowering stem is produced about 18 months after the isolation of the cutting. It is perhaps worth noting that all my attempts to find a single authenticated case in which the horseradish has produced mature seed have failed, nor can I trace a herbarium specimen with mature fruit. *A. rusticana* is suspected to be a polyploid variant of the Hungarian *A. macrocarpa*, but I have not succeeded in obtaining material of the latter for study.

6. POLARITY

In sharp contrast to callus-regenerating roots such as seakale, my observations suggest that horseradish exhibits little polarity. Of the differentiated primordia, roots are fairly evenly distributed along the whole of the cutting; buds are more frequent towards the proximal end, the proximal half of a cutting normally bearing about twice as many buds as the distal half. Over the cutting as a whole roots and buds are approximately equal in number, provided the cutting exceeds about 2 cm. in length; in shorter lengths the bud/root ratio rises sharply. This effect will be considered later in connexion with observations on correlation phenomena.

These observations are strikingly at variance with those of Lindner (1939), whose material was markedly polar. In the first place, however, it must be remembered that the old roots used by Lindner differed in other characteristics: notably, they readily formed callus on the cut surfaces, a phenomenon I have never seen in young roots regenerating for the first time. In this respect, old roots somewhat resemble seakale in behaviour; they may possibly further resemble it in an increased polarity. There is also a possibility of geotropic effects. The roots used here were allowed to regenerate in a horizontal position; it is possible (though not explicitly stated by him) that Lindner's roots were vertical. This possibility is still under investigation.

Extension of differentiated buds is, even in young material, strikingly polar. On cuttings between 2 and 14 cm. long, only one bud will normally grow out into a new crown; and this is always a bud very close to the proximal end of the cutting.

7. DISCUSSION

(*a*) *Ripeness to regenerate*

If detached horseradish root is to be able to regenerate it must have reached a certain stage of development, which may conveniently be called 'ripeness to regenerate'. Before new primordia will form on a detached root cutting, the primary cortex must have been shed and some secondary tissue developed. Roots reaching this stage are of the order of 2 months old, but the anatomical state of the plant gives a more reliable indication of the age at which regeneration becomes possible. Roots still attached to a crown will not, however, regenerate *in situ* until very much later; this is a correlation phenomenon, and will be considered in a later paper.

(*b*) *The changing function of the pericycle*

The versatility of the region usually known as 'pericycle' has been remarked before: by Lindner (1939) for horseradish, and by Priestley and Swingle (1929) for other plants producing root-buds. It is only necessary here to note that in practically all dicotyledonous roots the pericycle has two special functions: within it arise both the lateral root primordia and the phellogen. In plants such as horseradish and (Emery, 1954) *Chamaenerion* it has a third; for the new primordia, formed as they are from the phellogen, may be regarded as pericyclic in origin.

(*c*) *Limitation of function of the pericycle*

The phellogen is not universally capable of producing buds and secondary roots (except under very special conditions of auxin treatment), as these are produced only where the tissues derived from the pericycle have been penetrated by a primary lateral root trace. An explanation for the limitation of pericyclic activity cannot be deduced directly from the evidence presented here. As far as the author is aware, no previous studies have been made on such a close association of bud and lateral root trace. Cases of the existence of a similar phenomenon have been noted (Priestley and Swingle, 1929, *Statice limonium*), but no explanations have been suggested. It is perhaps not entirely fanciful to note that the association bears a curious resemblance to the association between a leaf and its axillary bud.

The effect is not simply that of penetration: new primordia do not arise beside a wound made by a needle-point. A possible physical explanation, which could perhaps be investigated, is that the flow of nutrients or special substances along the lateral root lowers the 'surface tension' of the malleable meristematic tissues around the scar when the primary lateral root is removed. Wardlaw (1952) refers to theories of normal bud initiation where the fixed position of a new bud is possibly due to lowered surface tension on the plant apex at the site of bud origin. Alternatively, buds and secondary roots could be directly promoted by the diffusion of specific bud- or root-forming substances from the old root trace. In this connexion the effect of vascular tissue on cell-division in tissue-culture (Jablonski and Skoog, 1954) is possibly relevant.

(*d*) *The indifferent apex*

Perhaps the most important feature to be noted from the examination of the morphological and anatomical evidence is that a generalized meristem can become either a bud or a root. Physiological evidence of the existence of the indifferent apex will also be presented in later papers. For example, the submergence of a cutting in water causes the proportion of bud to root primordia to change, whilst the total number of primordia remains constant. This could only occur if primordia were not predetermined in the direction of their growth.

Phenomena of a similar nature, but involving a direct transformation of a root apex into a bud or the reverse transformation, are widespread throughout the literature. Hölle (1875) described indifferent apices in *Ophioglossum*, and Goebel (1878, 1900) noted the spontaneous change of a root growing-point into a shoot growing-point in *Anthurium longifolium* and *Neottia nidus-avis*. Goebel stated that 'the transformation of roots into shoots is in my opinion only an individual case of the general phenomenon that shoots arise upon roots' ('Organography', 1900). Rostowzew (1890) examined transformations of root to stem in *Asplenium esculentum* and four species of *Platycerium*, and Howard (1938) found that *Brassica oleracea* stems could, when treated with 1 per cent. indoleacetic acid, be induced to form roots or buds from meristems produced near the vascular bundles, and in some cases buds or parts of buds were found to develop as roots. This case is closely paralleled by the indifferent apex in horseradish, and by the observations of Emery (1954) on *Chamaenerion*, which has been shown to develop meristematic masses on its roots which give rise to buds or roots.

Beijerinck (1887) found that in *Rumex acetosella* buds became roots if a cutting was placed with one end in the soil. Edmondson (1925) confirmed this, and she figures a young regenerated root showing a scale leaf at its base. Now if *Rumex* develops in a like manner to horseradish, but remains longer in an indeterminate state, a situation could arise where the rim of the hemisphere starts to develop as a leaf, but where this growth is stopped by changed conditions which cause the dome of the hemisphere to lose its dormancy and grow out as a conical root apex. The rim of the hemisphere would remain at the base of the root as a scale leaf.

Wardlaw (1952), commenting on Lindner's (1939) work, hints of the possibility of the formation of roots on horseradish in regions where buds would otherwise be formed; he says, 'the growth-regulating substance apparently has a more or less direct effect on the organisation of the meristem of a root or of a shoot in different instances'. Wardlaw also acknowledges the existence of undetermined meristematic tissue in ferns.

It is of course possible that some of the early claims have arisen from inadequate observation, a view expressed by Bloch (1943). Nevertheless, it seems on balance that Goebel was probably correct in regarding such transformations as not especially unusual; and it seems reasonable to add the regenerated primordia of horseradish to the list of indifferent organs. It is, however, surprising that so little attention has been given in recent years to such structures; they are surely very promising materials for the study of morphogenesis.

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Chilling and Flower-bud Stimulation in Pyrethrum (*Chrysanthemum cinerariaefolium*)

BY

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With three Figures in Text

ABSTRACT

The flowering of pyrethrum at the normal altitudes at which it is cultivated in East Africa depends on the amount of chilling available for the earlier stimulation of the flower-buds.

The period of maximum flower production follows some 3 months after the period of maximum cold in the cold season.

Attempts were made to delay the period of maximum flower production by cutting back new growth. These failed because chilling was so intimately connected with flower-bud development.

A tentative upper limit of temperature for flower production in East Africa varieties has been selected.

INTRODUCTION

THE pyrethrum plant grows well in the cool highlands of East Africa at altitudes between 6,000 and 9,000 ft. above sea-level. Occasionally it may be encountered outside this zone, but in general the present varieties do not flower well at altitudes much below this. It grows vigorously throughout the year, given sufficient rain. Flowering in the equatorial highlands starts about May or June, and reaches its maximum, 'the flush', in the period September–January, depending to some extent upon altitude. Thereafter the yield of flowers decreases rapidly, and the plant passes through a 'dormant' phase in the latter half of the 'hot season' (Jan.–April). During the dormant period vegetative growth continues if the water-supply is adequate to maintain it, but bud development is markedly suppressed. Small flushes may sometimes occur in the period June–September and, if the hot season is unusually cold, flowering may be prolonged beyond its usual limits. A typical flowering season at Molo in the Kenya Highlands (8,300 ft.—0° 14′ S.) is shown in Fig. 1.

During an investigation of the relationship between climate and the prevalence of a fungus (*Ramularia bellunensis*), which attacks and kills the immature flower-buds in most of the main growing areas, it was observed that bud-production was related to air temperatures which prevailed some weeks earlier. In particular it was noticed that cold spells were followed by an increase in the number of flowers harvested some weeks later. This observation was of considerable practical importance, for it had a bearing on a cultural practice which had been suggested by Gaddum (1947) and others as

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a means of avoiding the worst of the disease. It was therefore investigated in some detail.

Martin and Tattersfield (1934) have described chilling effects in pyrethrum grown in England, and Schwabe (1950) has discussed the importance of chilling in other species of Chrysanthemum. Both were concerned with experiments on a scale suited to laboratory and greenhouse.

As the production and control of cold in the tropics requires more expensive equipment than that for the production and control of heat in more temperate climates, laboratory studies could not be carried out on a large scale. This paper is therefore mainly concerned with the results of field experiments.

ANALYSIS OF FIELD DATA

The first hint that chilling was intimately connected with bud-stimulation was given in the results of an experiment conducted at the Pyrethrum Experiment Station, Molo. There a weekly record of the number of flowers and diseased buds harvested from twenty clonal plants was maintained from May 27 to December 13, 1950. As the station also records temperature and humidity measurements in a Stevenson screen near the experimental plots, daily records were available for examination. It was already obvious that diseased buds had been infected some time before they had been harvested, so the climatic conditions responsible for the flare-up in disease were sought at an earlier date than that of the harvests.

When the yield, temperature, humidity, rainfall, and other data were graphed, it was observed that there was an inverse relationship between mean maximum air temperatures and the numbers of flowers and diseased buds harvested 12 weeks later. The relevant data are shown in Fig. 1 (*a*). For convenience the mean maximum temperatures are shown in the inverted position. Except for the first few weeks of the season, the agreement is remarkably close and the regression of yield on temperature is very highly significant. The explanation of the apparent discrepancy early in the season was sought in the numbers of hours during which the plants were exposed to temperatures at or below certain selected levels. Of these, the hours spent at or below 60° F. gave the best agreement with the yield data. They are shown by the interrupted line in Fig. 1 (*b*). The relationship is, of course, a direct one: yield increases as the hours of exposure to temperatures at or below 60° F. increases. The marked differences in the early season have been mainly eliminated by this method, thus suggesting that although mean maximum temperatures at this time were high, there was sufficient chilling to stimulate bud development.

EXPERIMENTAL TREATMENTS

The chilling stimulus was further investigated at this station under controlled conditions. At the end of the dormant season in April 1952, when bud growth was active once more, ten closely similar large plants were

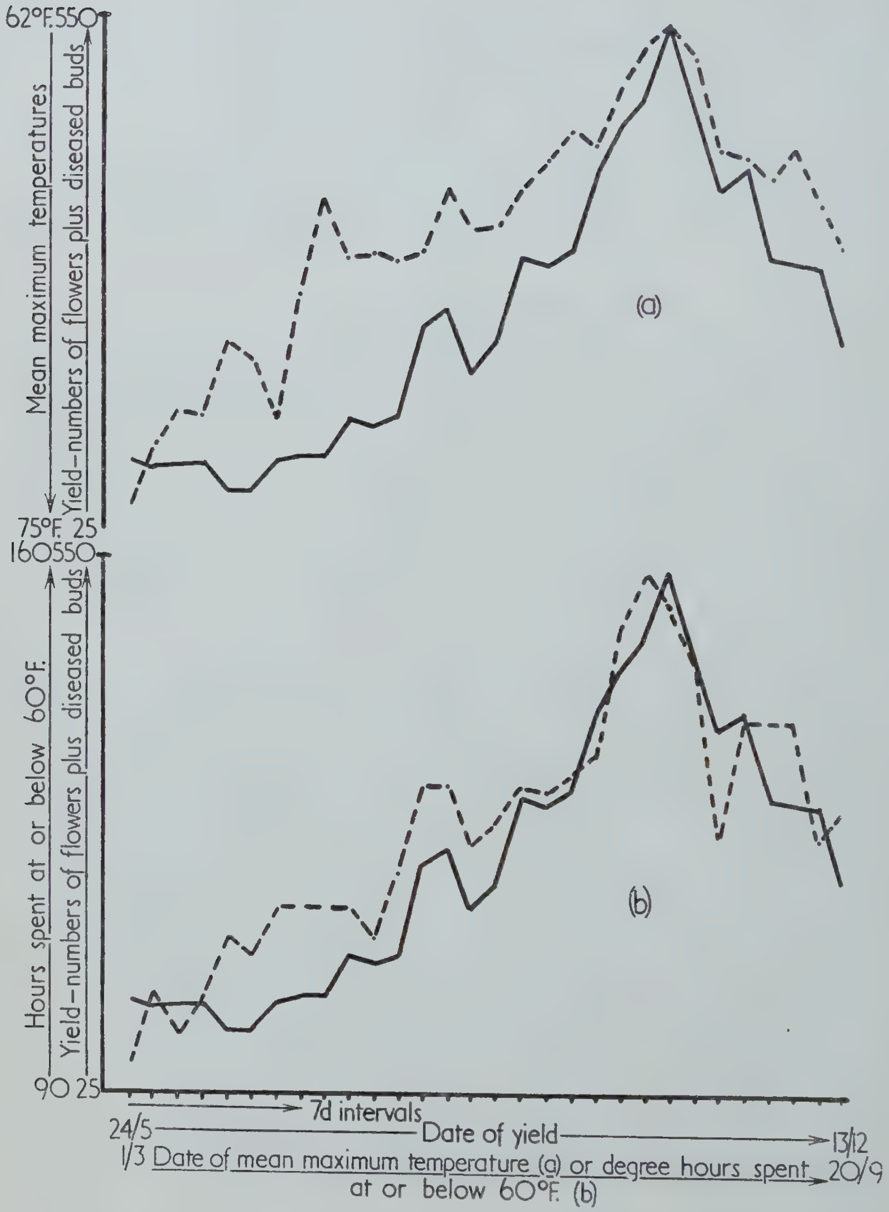
62°F. 550
Mean maximum temperatures
Yield—numbers of flowers plus diseased buds
(a)
75°F. 25
160 550
Hours spent at or below 60°F.
Yield—numbers of flowers plus diseased buds
(b)
90 25
7d intervals
24/5
Date of yield
13/12
1/3
Date of mean maximum temperature (a) or degree hours spent at or below 60°F. (b)
20/9

FIG. 1

selected from a series grown in pails out of doors. Although the whole series was reputedly clonal, these particular plants had not flowered in 1951, and unlike the remainder had shown no bud growth at this time. They were typical of the 'blind' plants encountered in commercial fields in that they were diageotropic in habit. A small well-lit cold chamber was available, and four of the plants, selected at random, were placed in this for 10 days. During this period the chamber was controlled at 60° F. and 60 to 80 per cent. relative humidity. The remaining six plants were exposed in the open and during the 10-day period spent an average of 9 hours a night at or below 60° F. Every night the minimum temperature fell to at least 53° F., and on 3 nights during the period, 5, 3, and 6 hours respectively were spent below 50° F. During the day an average of 6 hours per day was spent above 68° F. Air humidity ranged between 35 and 100 per cent. R.H.

After exposure in the cold chamber the plants were removed and placed with the controls. All were then left side by side in the open and bud development was watched. Within 6 weeks the four plants from the cold chamber showed marked bud growth as compared with the others. At the end of 3 months they bore 90 mature flowers compared with only 5 on the untreated plants. Furthermore, all of the four treated plants bore flowers, whereas only two of the six untreated plants had flowered. At the end of 5 months the treated plants had yielded a further 385 flowers, which was about the average yield for similar plants during this period; while the untreated ones had produced only a further 92, which was much below average. A χ^2 test showed the differences were very significant.

In the succeeding year the treatment was repeated, but this time four of the untreated control plants were chilled in the cold chamber, and the four previously treated plants, together with the remaining untreated plants, were left as controls. There was a possibility that the four previously treated plants, now used as controls, would carry over the effects of their earlier chilling. Environmental conditions were very similar, and in the beginning similar results were obtained. The treated plants again outyielded the controls.

However, later in the year, 5 months after treatment, the untreated plants, particularly the four which had been treated in the previous year, showed signs of catching up in yield. Over the last 5 months of the flowering season, September–January, they consistently yielded a few flowers more than the treated plants, and this difference was significant at the 5 per cent. level. The reason for this is not known. The difference may be fortuitous, or it may be due to slight differences of exposure. On the other hand, it may be due to a slight carry over of chilling from the previous year, which reinforces the effect produced by the normal seasonal chilling. The experiment is too slight to lead to any satisfactory conclusion, and more work is required to settle the point. The results of these two experiments seem to show that persistent chilling, even if only slight in terms of temperature, is more effective in producing bud-stimulation than more severe chilling maintained intermittently at night and alternating with periods of higher day temperatures.

CROP DATA

As all the aforementioned experiments were on a small scale, using only clonal material, it was decided to examine the relationship between large commercial yields and mean monthly maximum temperatures throughout the year. In this way the possible chance that only one special variety reacted to chilling could be eliminated, for the yields from large farms include the yields from plants of different varieties and different ages.

As most growers do not keep temperature records, two farms were chosen for their nearness to meteorological stations. One, in Kenya, was beside such a station; the other, in Tanganyika, was at several hundred feet higher than the station, but subject to similar climatic changes. The results of the comparison in the same year (1950) are shown in Fig. 2 (*a*) and (*b*). Again, for convenience, the mean maximum temperatures are shown in the inverted position and displaced in time. No numerical values for temperature are shown in Fig. 2 (*b*) because of the difference in altitude between farm and meteorological station. The figures show clearly that the effect of chilling on later yield is well marked on both farms, and that the flush in the same year occurs at the same time of year, although both are separated by some 3° 35′ of latitude.

At this stage the evidence for the importance of chilling as a major factor in bud development seemed fairly conclusive, for neither length of day nor rainfall nor soil differences could seriously be considered as affecting the plants. If photoperiodism were effective, then the varieties in use in East Africa would have to be very sensitive indeed, for in one of the main growing regions in the highlands of Kenya day-length varies only by some 2 minutes throughout the year. Rainfall, too, is not a controlling factor, for in certain areas of Tanganyika the flush occurs towards the end of the dry season after several months of very low and unreliable rain, whereas in parts of Kenya it follows the wet season. Of course, serious lack of rainfall can influence flower production in that drought will not permit the normal growth of the buds. Soil differences, likewise, are not important. Differences in fertility and structure may affect the vigour of the plant; but plants growing on very different soils all flower at the same time of year.

If chilling is as important as it seems, then, in a normal season in East Africa where the stimulated buds grow quickly in the normal warm day temperatures, the time of maximum flower yield should be determined by the time of maximum cold, and this relationship should be difficult or even impossible to alter. This conclusion is of considerable practical importance, for if any cultural practice reduces the number of buds available for stimulation at the height of the cold season, then it should automatically reduce yield. Fortunately this could be tested immediately. It had been suggested by those interested in the disease problem that, if early buds and flower stalks were heavily cut back, then subsequent budding and maturation of flowers would take place later than usual, and the plants might avoid the climatic

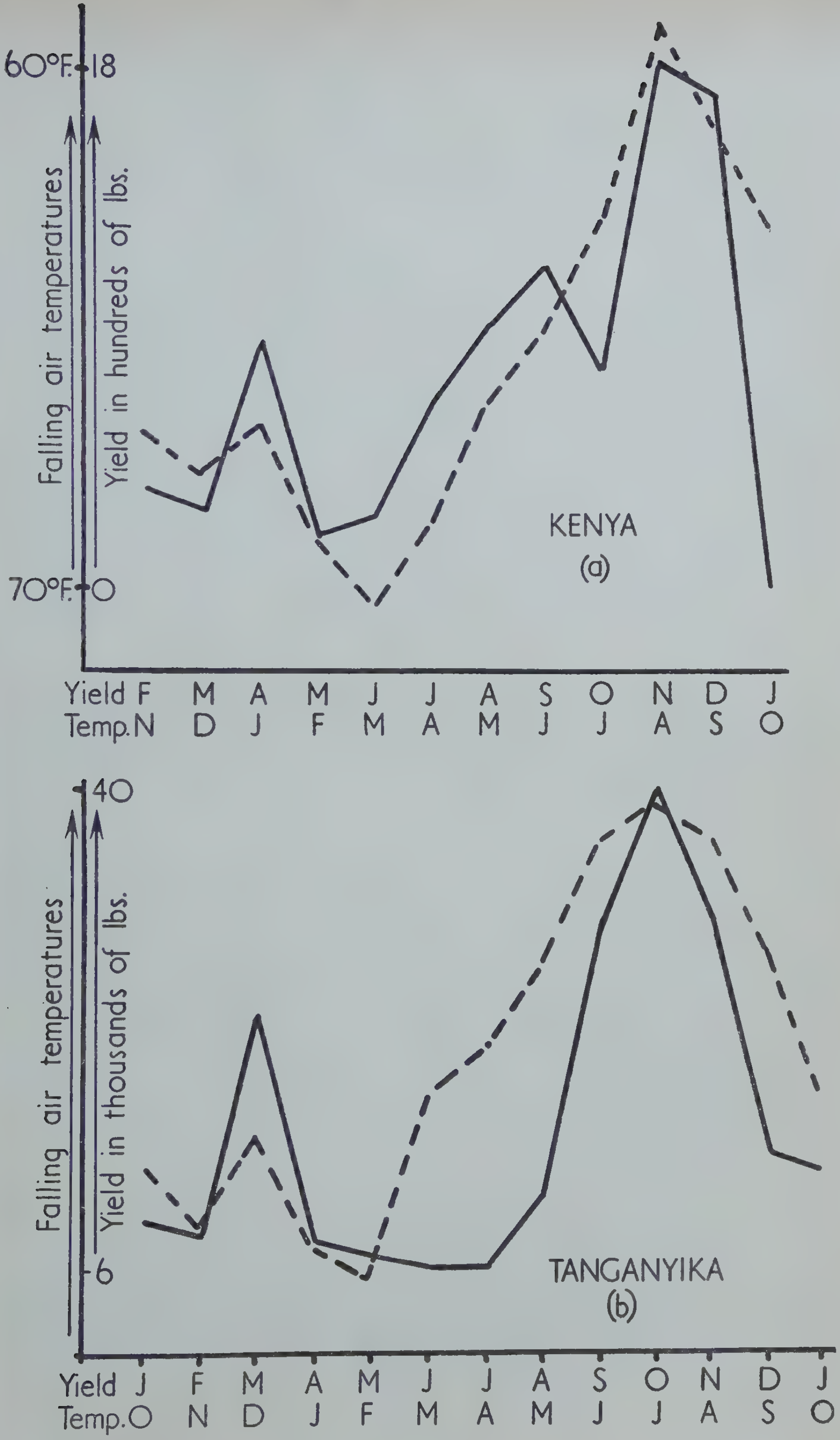
60°F.
18
70°F.
0
Falling air temperatures
Yield in hundreds of lbs.
KENYA
(a)
Yield F M A M J J A S O N D J
Temp. N D J F M A M J J A S O
40
6
Falling air temperatures
Yield in thousands of lbs.
TANGANYIKA
(b)
Yield J F M A M J J A S O N D J
Temp. O N D J F M A M J J A S O

Fig. 2

conditions which favoured the disease. Inherent in this suggestion was the assumption that such treatment would cause no serious loss in yield.

It is normal farming practice to cut away all the dead stalks and leaves to within 4 to 6 in. of the ground during the dormant season every year. This leaves the leafy, basal clump from which new flower-stalks arise. It makes picking easier in the subsequent season. Such a procedure was adopted in an experiment to find out the most suitable date for cutting back plants at Molo in 1949. There were 26 plots each of 10 clonal plants. They were divided into 13 groups of 2 by random selection. One group, the control, was left uncut after the previous year's harvest. One each of the remaining 12 groups was cut back on the first of each month from February to January of the succeeding year. Thus progressively larger amounts of new growth were removed at each cutting. In this manner material which had been cut back at the beginning of every month of the year was available for comparison with the controls. The numbers of flowers and large diseased buds produced by each group of plots were counted every 10 days throughout the flowering season. A selection of the results is shown in Fig. 3 (*a*)–(*f*). In each small figure the continuous line represents the control yield (numbers of flowers+diseased buds) and the interrupted line shows the corresponding yield from the treated plots.

Figs. 3 (*a*) and (*f*) illustrate the remarkable regularity of behaviour between different groups of plots. Group (*a*) shows that early cutting-back does not affect total yield or even the size of the harvest every 10 days. Group (*f*) shows that, as might be expected, very late cutting back in the middle of the flush merely causes the loss of the remainder of the crop.

Figs. 3 (*b*), (*c*), (*d*), and (*e*) show two important things. One is that when the plant recovers its production after cutting back the yield only rises to the level of the control yield and not, as might be expected, to a higher level. The other is that once this recovery is made the yield of the cut-back plants rises and falls in the same manner and to the same extent as the control yield. Both (*c*) and (*d*) show the marked fall in yield recorded on October 8. In the case of (*c*) it was after the treated plants had reached the levels of the controls. In the case of (*d*) it was while the plants were recovering to the control level. A similar effect to that of (*d*) is shown in November in (*e*). It is obvious that such remarkable behaviour is unlikely to be influenced by any internal factor and that only an external factor in the environment of the plants could produce it. Since chilling has already been demonstrated to be a very important factor in bud-stimulation, it seems reasonable to conclude that it is responsible for the plant behaviour in this experiment. Once the plants had recovered from the losses due to cutting back their subsequent yield was determined, in a like manner to that of the controls, by the amount of chilling they received. The result of this experiment suggests that it is impossible to displace the flush in time by cutting back new growth; and that such a procedure would merely cause loss in yield if too long delayed, and would not enable the plant to avoid the disease.

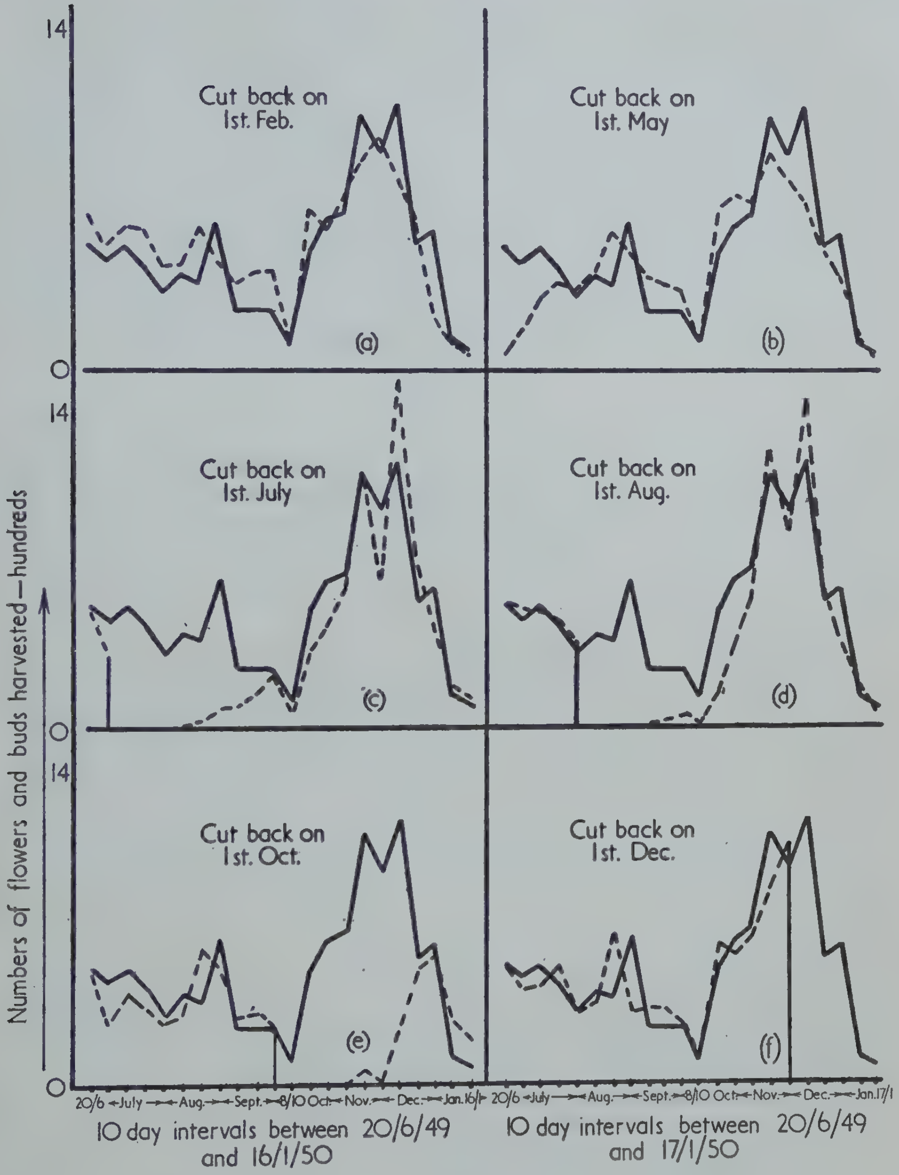

FIG. 3

Although the threshold requirements of chilling are not known, it is possible to obtain an estimate of the mean maximum temperature in the Kenya Highlands which, if prolonged, leads to little or no subsequent yield of flowers in the local varieties. This estimate may be derived from the regression equations of yield on earlier mean maximum temperatures. Such equations have been calculated for three different varieties in three different years and at two widely separated sites. At this station (6,800 ft. a.s.l.) in 1952 the yield of one variety was likely to be nil when earlier weekly mean maxima were 73° F. or higher. At Molo in 1949 the yield of another variety was likely to be nil when earlier 10-day mean maxima were 72° F. or higher. Again, at Molo in 1950 the yield of yet another variety was likely to be nil when weekly mean maxima were 75° F. or higher. The agreement between these results is remarkable. It would seem that when mean maxima of this order occur in the Kenya Highlands the range of temperatures associated with them are such that any night chilling is largely ineffective. One might therefore select as a tentative upper limit the maximum temperature 75° F. which, if it persists for a week or more, greatly inhibits flowering in the East African varieties.

DISCUSSION

The results of the experiments show that chilling of the buds is essential to the development of flowers. The cold-box experiments show that under our short-day the required time of exposure is short. Only 240 hours at 60° F. was sufficient to set in motion the normal production of flowers in blind plants. This time is somewhat shorter than that reported by Schwabe (1950) for ornamental Chrysanthemums.

One remarkable feature of the growth of pyrethrum in East Africa is the long flowering season, compared with the short seasons which Gnadinger (1945) reports as normal in Yugoslavia and Japan, the other main growing areas. It would seem that the slow increase in cold throughout the season in the constant short-day of Kenya is responsible for the slow increase in yield and the prolongation of the flowering season, whereas the more intense cold of winter and early spring in Yugoslavia and Japan, followed by the rapidly increasing warmth of spring and early summer, may be in part responsible for the rapid and complete development of most of the buds to make a short flowering season.

In the latter case it would seem that all or nearly all the flower-bud primordia are laid down during or after flowering in one year, and are all stimulated together in the next year. A similar situation may occur in Kenya, but if it does it is not obvious why certain buds should be stimulated and others not, unless the stimulus is required at a particular stage of bud development. In this case the primary buds would have to be at a stage suitable for stimulus, whereas the secondaries would require to reach that stage before chilling becomes effective. A more likely explanation of the long season lies in the fact that vegetative growth hardly ever suffers a check. New flower-buds develop on new vegetative shoots which continue to develop throughout the flowering season. The production from these is reinforced by that from the development, and further branching, of some lateral shoots on the primary flower-stalk after the first flowers are removed in early pickings. There is therefore a continuous succession of buds being differentiated throughout most of the year, and these in turn are subject to stimulation.

The detection and subsequent confirmation of the importance of chilling and the selection of a tentative upper limit of maximum temperature suited to the growth of East African pyrethrum varieties was made easier by the particular climate of the highland regions. As is shown in Fig. 1 (*a*), the mean maximum air temperature changes slowly throughout the year. The mean minima tend to remain remarkably constant. For example, during the period shown in Fig. 1 (*a*) the mean minima ranged from 41 to 51° F. and 90 per cent. of the records were in the range 41 to 47° F. There was no marked seasonal trend as for the mean maxima. Although night temperatures are low, day temperatures are high, and there is little or no check in growth once the buds are stimulated.

One aspect of the work has not been touched upon for lack of sufficient data. It has been observed that the yield of some farms above 9,000 ft. sometimes resembles those that were studied at the more usual lower altitudes, and sometimes differs. Unfortunately such farms are not near stations which record temperature. In the only case which could be investigated it seemed that chilling was always adequate at such altitudes, and that subsequent flushes of flowers were determined by the presence of sufficient warmth to permit rapid growth.

SUMMARY

Field experiments show that the yield of pyrethrum flowers in East Africa is inversely related to mean maximum temperatures, and directly related to the number of hours spent by the plant at or below 60° F., some three months earlier. They suggest that chilling is an essential part of flower-bud development.

Experiments with 'blind' plants in a cold-box show that 240 hours' continuous exposure at 60° F. is sufficient to stimulate the development of flower-buds, and less may be adequate.

Attempts were made to delay the period of maximum flower production by cutting back new growth. These failed because chilling was so intimately connected with flower-bud development. The removal of buds which would normally be stimulated at the period of greatest cold merely reduced subsequent yield. There was no compensating increase in yield at a later date.

The temperature régime of the Kenya highlands is such that it is possible to select 75° F. as a tentative upper limit of mean maximum temperature which, if prolonged for a week or more, leads to the inhibition of flower production. It is the range of temperatures associated with this maximum which probably produces the inhibition, not the temperature itself.

ACKNOWLEDGEMENTS

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The Effect of Certain Adsorbents on the Nodulation of Clover Plants

BY

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ABSTRACT

The addition of charcoal to the rooting medium of clover plants inoculated with effective and ineffective strains of *Rhizobium* leads to a stimulation in nodule production. The time interval between inoculation and the first appearance of the nodules is reduced in the presence of charcoal. Several explanations of this phenomenon have been investigated and it is concluded that the stimulation is due to the adsorption by the charcoal of inhibitory compounds secreted by the clover roots. These compounds have been eluted from the charcoal and have been shown to affect nodule production. As a result of these experiments it is suggested that, depending on the concentration of the secretions in the eluates, either a stimulation or inhibition of nodule production may occur.

Other adsorbents have also been added to the clover plants, but no consistent stimulation of nodule production has been observed.

INTRODUCTION

THE improved growth of plants following the addition of charcoal to the rooting medium has been ascribed to the adsorption of toxic compounds secreted by the roots of the plant (Zinzadze, 1932; Papadakis, 1941); to an improvement in the growing conditions, e.g. by improved aeration of the soil (Gukova and Butkevich, 1941; Tincker, 1947); or to the maintenance of a favourable pH in the neighbourhood of the roots (Prianishnikov and Domontovitch, 1926). It seems unlikely, however, that the small amounts of charcoal used could materially alter the aeration or that the buffering action of the charcoal is sufficient to explain the results on these grounds.

Preliminary results by Nutman (1952) demonstrated that the addition of charcoal, bentonite, or fuller's earth led to an increase in the nodule numbers on clover plants (*Trifolium pratense* L.) inoculated with *Rhizobium* strains. These results supported an earlier conclusion of Nutman (1945) that nodulation was influenced by root secretions from the plants.

Vantasis and Bond (1950) obtained an increase in dry weight and in the amount of nitrogen fixed by the addition of charcoal to inoculated pea plants (*Pisum arvense* L.) growing in sand culture. They considered the possibility that charcoal might supply minor trace-elements, but concluded that the effect was due either to the adsorption of toxic compounds secreted from the plant or from the micro-organisms, or to the maintenance of improved growing conditions in the neighbourhood of the roots.

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Considerable experimental evidence, reviewed by Loehwing (1937) and by Gray and Bonner (1952), suggests that organic compounds secreted from the roots may affect nearby plants of the same or different species. But the evidence remains inconclusive until the secretions have been isolated and shown not to be due to the breakdown of dead plant material or to the leaching of organic material from the leaves.

Claims to have demonstrated the presence of root secretions from clover plants include those of Virtanen and Laine (1939), who showed that aspartic acid, β-alanine, oxime nitrogen, and fumaric acid may be secreted from the nodule-bearing roots of clover plants which are actively fixing nitrogen. On one occasion (Virtanen, 1948) glutamic acid was also identified. The influence of these compounds on nodulation has not been investigated.

Repeated cultivation of clover crops in the same soil may lead to 'clover sickness'. It has been suggested that this is due to the accumulation of toxic compounds in the soil secreted from the clover plants, since this disease may be alleviated by the addition of charcoal to the soil (Rothamsted Annual Reports, 1946, 1951).

Thornton (1929) demonstrated that nodulation on lucerne seedlings (*Medicago sativa* L.) was accelerated by the presence of other lucerne plants or by the addition of the liquid in which other lucerne plants had been grown. This result was confirmed by Ludwig and Allison (1935) and by Nutman (1953). In the latter paper Nutman discussed the interaction on nodulation of several leguminous plants and reported that when older plants were used an inhibition of nodule production occurred. These effects were attributed to the presence of root secretions, and it is possible that the varying response may be related to the concentration of these secretions. It should be noted that in the experiments of Vantasis and Bond (1950) the addition of charcoal to peas led to a decrease in nodule numbers, whilst in the experiments of Nutman with clover plants an increase in nodule numbers was reported.

In the present series of experiments the effect of added adsorbents on nodulation on clover plants has been investigated further.

MATERIALS AND METHODS

Clover plants were grown under sterile conditions on slopes of mineral salt aga medium in test-tubes (size 6 in. × $\frac{3}{4}$ in.) plugged with cotton-wool. The composition of the nitrogen-free agar medium has been described by Thornton (1930).

The seed used was either commercial late-flowering 'Red Montgomery Clover' or seed of selected lines which had been raised from commercial seed by selection for earliness or lateness in time of nodulation and abundant or sparse numbers of nodules (Nutman, 1946).

Surface sterilization and softening of the seed-coat was carried out by immersing the seed in concentrated sulphuric acid for 30 minutes followed by thorough washing in sterile distilled water. One seed was sown per tube and germinated in the dark for 2 days. Two ml. of sterile nitrogen-deficient nutrient solution (Thornton, 1930) were added containing the inoculum of *Rhizobium* strains which were known to be fairly stable as regards the number and effectivity of nodules produced.

The 2-ml. level in the tubes was marked and during the course of the experiments the level of liquid in the tube was periodically adjusted to the mark by adding sterile nutrient solution. The plants were kept in racks so that the roots were shaded and were kept in the greenhouse. The plants were usually harvested after 10 to 12 weeks' growth.

Clover plants were also grown in boiling-tubes (size 7 in. × 1½ in.). Two plants were grown in each tube, the volume of liquid per tube (6 ml.) being such that no reduction in the number of nodules on the plants occurred through plant interaction.

Quantities of the adsorbent were weighed out into small narrow tubes (size 5 in. × ½ in.), dry sterilized by heating to 150° C. for 10 hours, and added to the plants at the time of inoculation.

The commercial bentonite was cleaned by differential centrifugation. A thick suspension of bentonite in water was centrifuged at 3,000 r.p.m. for 2 minutes. The precipitate consisting of black and grey particles was discarded and the supernatant was spun at 4,000–5,000 r.p.m. for 15 minutes. The precipitate consisting of a pale yellow nearly homogeneous clay was dried at 75° C. and powdered. This powder was added to the plants as described above.

In the initial experiments commercial high-grade charcoal was purified as described by Brown et al. (1951). This charcoal, however, proved toxic to the clover plants, withering of the petioles and discoloration of the leaves occurring when the plants were a few weeks old. By treating the charcoal successively with boiling N. sodium hydroxide, boiling 10 per cent. (v/v) hydrochloric acid and hot water followed by thorough washing with cold distilled water, it was possible to obtain a sample of charcoal showing no toxicity to the clover plants. Later experiments indicated that the toxic compounds were removed during the alkali treatment which was shown to remove quantities of silica and aluminium from the charcoal. However, the addition of pure silica to the clover plants gave rise to no toxic symptoms.

EXPERIMENTAL RESULTS

The stimulating effect of charcoal on nodule numbers was confirmed in the present series of experiments as shown in Table I below. The charcoal had no effect on the initial or final pH of the added nutrient solution. The effect of charcoal on the time interval between inoculation and the appearance of the first nodules on the roots is shown in Table II. With both effective and ineffective strains of *Rhizobium*, the presence of charcoal has decreased this time interval. Since all plants were harvested on the same day, those in contact with the charcoal were producing nodules over a slightly longer period

TABLE I

The Effect of Charcoal on Nodule Numbers of Clover Plants

Experiment.	Treatment.	Average number of nodules per plant 12 weeks after inoculation.
Commercial seed Inoculated with clover strain F.	Control, no charcoal added	(Average of 48 plants) 22·3; 27·6
Plants growing in pairs in boiling tubes	Plus 0·5 g. charcoal per tube	48; 38; 45; 58
		(Average of 20 plants)
Plants grown singly in test-tubes	Control, no charcoal	19·0; 19·4
Commercial seed	Plus 0·1 g. charcoal	36·4; 43
Plants grown singly in test-tubes		
Seed 331 × 403	Control, no charcoal	16·3
	Plus 0·1 g. charcoal	53·9
Seed 57 × 52	Control, no charcoal	20
	Plus 0·1 g. charcoal	34
	Plus 0·2 g. charcoal	36
Seed 76 × 80		
Inoculated with ineffective strain H.K.C.	Control, no charcoal	27 (after 39 days)
	Plus 0·1 g. charcoal	48 (after 39 days)

TABLE II

The Effect of Charcoal on the Time Interval between Inoculation and the Appearance of the First Nodule

Experiment.	Treatment.	Average time interval, days. (Average of 20 plants)	Significance of difference between means. (P 0·05, $t = 2·0$)
Family 76 × 80	Control, no charcoal	16·6	—
Inoculated with ineffective strain H.K.C.	Plus 0·1 g. charcoal	10·5	2·65
Family 63 × 66	Control, no charcoal	15·5	—
Inoculated with effective clover strain	Plus 0·1 g. charcoal	12·9	2·0

(i.e. 3–4 days) than those plants having no added charcoal. From the data of Nutman (1948) the rate of nodulation for an abundantly nodulating line of clover plants is approximately 0·5 nodule per day over 98 days. Consequently the large stimulation in nodule number by the charcoal cannot be ascribed to the decrease in the time interval required for nodulation to occur. However, in short-term experiments (i.e. 10 days) with plants inoculated with ineffective strains of bacteria, the number of nodules would appear to be related to the number of days since nodulation first occurred (Table III). This correlation, however, is not evident at the time of harvest (i.e. 39 days after germination).

Addition of ashed charcoal to the clover plants caused no increase in nodule numbers. Twenty grammes of purified charcoal were heated in a muffle furnace and yielded 2 g. of ash. 0·01 gm. of ash (which corresponded to 0·1 g. of purified charcoal normally added to the test-tubes) and 0·1 g. of ashed charcoal (which corresponded to the same volume as the 0·1 g. purified charcoal) were added to the plants and the subsequent effects on nodule numbers are recorded in Table IV. No increase in nodule numbers occurred.

TABLE III

Rate of Nodulation on Clover Plants inoculated with Ineffective Rhizobium Strains

Experiment.	Treatment.	Average of 20 plants. Days since nodulation.	 Average nodule number per plant.	 Increase in nodules *per diem.*
Seed 76 × 80 Inoculated with ineffective strain	Control-nutrient solution only	10	10	1·0
	Eluate from charcoal	9	10	1·1
	Plus charcoal 1	14	14	1·0
	2	11	13	1·2
	3	16	18	1·1
	4	14	12	0·9
	Plus bentonite	12	12	1·0

All plants were sown and later counted on the same day.

TABLE IV

The Effect of the Addition of Ashed Charcoal on Nodule Numbers

Experiment.	Treatment.	Average nodule number per plant at harvest (20 plants per treatment).	
Seed 57 × 52	Control, no charcoal	22	
	Plus ashed charcoal 0·01 g.	18	
	Plus 0·1 g.	20	
		(Differences not significant at P 0·05 level)	
Commercial seed			
1	Control, no charcoal	18·2	(P 0·05 t 2·0)
2	Plus ashed charcoal 0·1 g.	13·6	t calc. 0·63
3	Plus charcoal 0·1 g.	36·4	t calc. 3·64

As the addition of charcoal to the nutrient solution did not alter the pH, it seems unlikely that the stimulatory effect of charcoal is due either to the presence of inorganic nutrient elements in the charcoal or to the improvement of physical conditions in the nutrient solution.

These conclusions are supported by the fact that no stimulation in nodule numbers occurred when the plants were grown in culture solutions of varying composition. These solutions contained adequate amounts of known trace elements and the solutions differed in the ratios of the inorganic ions present (Table V).

TABLE V

The Effect of Different Nutrient Solutions on Nodulation on Clover Plants

Solution.	Average nodule numbers per plant (20 plants per sample).
Nutrient solution (Thornton, 1930)	18·1
Nutrient solution plus added trace nutrients including molybdenum	17·4
Crone's nutrient solution	15·4
Nutrient solution plus agar extract	21

(Differences between means not significant at the P 0·05 level)

In the culture solutions used, the possibility existed that the calcium initially present in the solution was precipitated as insoluble calcium phosphate. In view of the known influence of calcium on the nodulation in soy beans (Albrecht and Davis, 1929), calcium as calcium gluconate was added to the nutrient solution to ensure a supply of available calcium to the plants. However, no effect was noted on the time required for nodulation or on nodule numbers when clover plants were grown in these solutions (Table VI).

TABLE VI

Effect of Added Calcium Gluconate on Nodule Numbers

Treatment.		Average number of nodules per plant at harvest (20 plants per sample).	Average number of days to nodulation.
Seed 76×80 Inoculated with effective *Rhizobium* strains	Control	9·7	16
	Plus calcium gluconate	9·8	15

Pretreatment of the agar with charcoal or washing the agar slopes prior to the sowing of the seed did not prevent the subsequent stimulation when charcoal was added. Sterile agar slopes were heated to 100° C. Sterile charcoal lumps were added and the tubes shaken. The agar was then separated from the charcoal by decanting the liquid agar into sterile test-tubes. The agar was then sloped. To investigate the possibility that inhibitory compounds may be formed during the sterilization of the agar slopes, tubes after the initial pretreatment with the charcoal, as described above, were re-sterilized. In addition, sterile agar slopes were washed with sterile distilled water before sowing the seed.

In all treatments addition of charcoal to the plants led to a stimulation in nodule numbers (Table VII). Re-sterilization of the slopes after pretreatment with charcoal led to a small reduction in nodule numbers when charcoal was not subsequently added to the plants. Addition of the charcoal to these re-sterilized slopes gave the usual stimulation of nodule numbers. These results suggest that during the re-sterilization of the slopes a compound inhibitory to nodule formation may be formed which is adsorbed by further additions of charcoal. But it is improbable that the whole of the stimulatory effect of charcoal can be attributed to the adsorption of inhibitory compounds present in the agar. This conclusion was confirmed when it was proved possible to grow the clover plants in the absence of the agar slopes.

TABLE VII

The Effect of the Pretreatment of the Agar used in the Preparation of the Slopes on the Charcoal Stimulation of Nodule Numbers

Treatment.		Average number of nodules per plant at harvest (20 plants).	Significance of difference between means *P* 0·05, *t* 2·0.
Commercial seed inoculated with effective *Rhizobium* strains	1. Agar pretreated with charcoal, then used for slopes	20·4	—
	2. Agar pretreated as in 1. Additional charcoal added to the plants	30·9	—
	3. Agar pretreated with charcoal, then re-sterilized	14·9; 12·7	Between treatments 1 and 2, *t* calc. 2·8
	4. Agar prepared as in 3. Additional charcoal added to the plants	35·5; 44·2	Between treatments 1 and 3, *t* calc. 3·0
	5. Agar not pretreated	17·2; 21·5	Between treatments 1 and 4, *t* calc. 2·4
	6. Agar not pretreated, charcoal added to the plants	36·4	—
	7. Agar washed in distilled water for 48 hours before being made into slopes	19	—
	8. Agar as in 7. Charcoal added to the plants	43	—

Attempts were made to grow the plants in liquid culture and in various types of slopes, e.g. gelatine, bacterial cellulose, starch, and cilica gel, but in all cases the plants showed poor development and stunted root formation. Plants could, however, be grown satisfactorily on filter-paper slopes. These slopes were fitted into test-tubes, sterilized and moistened with sterile nutrient solution when the seeds were sown. Two days after germination 4 ml. of the nutrient solution were added containing the inoculum of *Rhizobium*. After

12 weeks' growth no difference in dry weight between the plants grown on agar and filter-paper slopes was found.

Results quoted in Table VIII indicate that the charcoal stimulation occurs when the plants are grown in the absence of agar.

The stimulatory effect of charcoal can therefore only be attributed either to the presence in the charcoal of an organic compound (which was destroyed by ashing) stimulating nodule production or to the adsorption by the charcoal of inhibitory compounds secreted by the plant. These possibilities were investigated further by studying the effect of elution by organic solvents on the charcoal before and after addition to the plants.

TABLE VIII

Effect of the Addition of Charcoal on Nodule Numbers in the Absence of Agar

Treatment.		Average nodule number at harvest (20 plants).	Significance between means P 0·05, $t = 2·0$
Commercial seed inoculated with clover strain F	Control, nutrient solution only	10·4	—
	Plus charcoal	20·5	3·13

Clover plants were grown in contact with charcoal and supplied with a pure culture of *Rhizobium* for 3 months. The plants were then harvested, the number of nodules counted, and the charcoal separated from gross contamination (i.e. plant debris) by filtering through a 1-mm. sieve. The charcoal was collected, washed well with distilled water, and dried at room temperature. Quantities of the charcoal were suspended in the organic solvents and left standing overnight at room temperature. The charcoal was filtered off and the solvent removed from the filtrate by vacuum distillation. The residue was taken up in sterile nutrient and stored at 0° C. until required.

Purified charcoal which had been suspended in nutrient solution containing agar extracts (but had not been used in the plant experiments) was also extracted as described above, this extract being used as control for the eluates from the plant-reacted charcoal.

To test the eluates, clover plants were grown on agar slopes and 2 ml. of the eluates inoculated with effective strains of *Rhizobium* were added. This volume was maintained throughout the experiment by further additions of the eluates as required. After 3 months' growth the plants were harvested and the nodule numbers and the dry weights of the plants were recorded.

Results given in Table IX indicate that compounds can be extracted from the charcoal which may influence nodulation on clover plants. The preparation of the eluates was identical in expts. 1 and 3 (Table IX). It may therefore be tentatively suggested that, depending on the concentration, one substance may be responsible either for stimulating or inhibiting nodule formation. In expt. 1 the difference between the means of the number of nodules per plant grown in nutrient solution and in the control charcoal eluate is not significant at the *P* 0·05 level. These results suggest therefore that the charcoal is adsorbing compounds secreted by the roots of the clover plants. But in view of the activity of the control charcoal eluates in influencing nodulation as shown in expt. 2, Table IX, the conclusions can only be regarded as tentative.

Table IX also shows the effect of the elutions on the dry weight of the plants at harvest. The effect of the alcohol-ether of the charcoal collected from the boiling tubes after being in contact with the plant roots is particularly interesting. Although the number of nodules per plant has been significantly increased by the eluate, there has been a significant decrease in the dry weight of the plants compared with the dry weight of the plants treated with the control charcoal eluates. Of the 19 plants treated with the plant-reacted charcoal eluate, 10 showed symptoms of nitrogen deficiency (leaf and petiole colour). There was no reason to suppose that the normally effective strain of bacteria had, by mutation, become ineffective, since there appeared to be no difference in the size and total number of nodules on the nitrogen-deficient plants compared with the healthy plants in the same rack, i.e. undergoing the same treatment. Compounds may therefore be present in the alcohol ether eluate of the plant-reacted charcoal which interfere or inhibit the actual nitrogen-fixation mechanism.

TABLE IX

Effect of Charcoal Eluates on Nodule Numbers on Clover Plants

	Average number of modules at harvest (20 plants).		
Experiment.	Control, nutrient solution only.	Eluate from plant reacted charcoal.	Eluate from control charcoal.
1. Charcoal eluted with 70% acetone.	23·6	37·1	28·2
Tested with commercial seed and inoculated with clover strain F.	25·6	34·1	—
		Differences between nutrient solution and control charcoal eluate not significant at *P* 0·05 level	
2. Charcoal eluted with ethyl alcohol-ether mixture	23·6	34·5	31·2
		Differences between nutrient solution and charcoal elutes significant at the *P* 0·05 level	
3. Charcoal eluted with 70% acetone. Tested with family 87 × 83	28·3	21·3	21·7
		Differences between charcoal elutes and nutrient solution significant at the *P* 0·05 level	
	Effect of alcohol-ether eluate on dry weight of the plants at harvest (mg.).		
	Control, nutrient solution.	Plant charcoal eluate.	Control charcoal eluate.
	49·55	33·5	49·6
		Significant difference between dry weight of the control and plant eluate samples	

Elution experiments have therefore shown that compounds may be excreted by the clover plants which may affect several aspects of the nodulation and nitrogen-fixation processes. It also appears that compounds can be extracted from unreacted charcoal which may also affect nodulation. It is conceivable that similar compounds may be present in soil (i.e. through the degradation of organic material), and this would give further impetus to the study of the interaction between plant and soil.

Experiments using Ineffective Strains of Rhizobium

Experiments involving the elution of compounds from charcoal placed in contact with the clover roots require an over-all period of 6 months. Progress in the identification of the root secretions would be increased if quicker assay methods were available to test the eluates. Tests were therefore carried out using ineffective strains of *Rhizobium* since the rate of nodulation on clover plants is faster with these strains.

TABLE X

The Effect of Charcoal on Nodule Numbers using Inoculations of Ineffective Strains of Rhizobium

	Average number of days between inoculation and appearance of the first nodule (20 plants).	Average number of nodules at harvest (39 days).	Significance of difference between mean P 0·05, t 2·0 nodule numbers.
1. Control, nutrient solution only	16·6	26·7	—
2. Plus charcoal	12·6	40·5	Between treatments 1 and 2, t 2·5
3. Plus acetone pre-treated charcoal	10·5	47·9	Between treatments 1 and 4, t 4·5
4. Acetone eluate of plant-reacted charcoal which had been pretreated with acetone	17·6	16·5	

Results shown in Table X indicate that in these experiments addition of charcoal again stimulated nodule production. Charcoal which had been pretreated with boiling acetone caused a further increase in nodule number; a result which confirms earlier observations that inhibitory compounds are present in the unreacted charcoal. However, elutes from the acetone-pre-treated charcoal after it had been in contact with the plant roots still caused a large depression in nodule numbers. These eluates must contain inhibitory compounds adsorbed by the charcoal from the plants.

Effects of adsorbents other than charcoal on nodulation

The effect of a number of adsorbents on nodulation is shown in Table XI. In no case has a consistent stimulation been obtained. Numerous experiments have been carried out using bentonite under different conditions, but only spasmodically has an effect on nodule numbers occurred. If bentonite causes no consistent stimulation in nodule numbers, then the compounds excreted by the roots which lead to the formation of coloured complexes on the surface of the bentonite first reported by Nutman (1951) and examined in greater detail by Turner (1954) are not identical with those which inhibit nodule formation.

TABLE XI

The Effect of the Addition of Other Adsorbents on Nodule Numbers

Treatment.	Average number of nodules at harvest (20 plants).
Seed 010	
Nutrient solution	28·7
Plus bentonite	28·0
Seed 76 × 80	
Nutrient solution	26
Plus bentonite	47
Commercial seed	
Nutrient solution	22·3; 27·6
Plus bentonite	34·9; 25
Seed 110	
Nutrient solution	20
Plus mica	20
Plus illite	18
Plus hectorite	14
Plus ferric oxide	21
Plus aluminium oxide	19

SUMMARY

1. The number of nodules on clover plants inoculated with effective or ineffective strains of *Rhizobium* is stimulated by the addition of charcoal to the rooting medium.

2. The stimulation is due to the adsorption by the charcoal of inhibitory compounds secreted by the plants.

3. The time interval between inoculation and the first appearance of the nodules is decreased in the presence of the charcoal.

4. Eluates obtained by the extraction of the reacted charcoal with organic solvents may either stimulate or inhibit nodule production. This effect may be related to the concentration of the root secretions in the eluates. Compounds may also be present in the eluate which interfere with the nitrogen fixation mechanism.

5. Compounds can also be extracted from charcoal which has not been in contact with clover roots, which may influence nodule production.

6. The addition of other adsorbents to the clover plants has not led to any consistent stimulation in nodule production.

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